# THE JOURNAL OF PHYSIOLOGY

EDITED FOR

## THE PHYSIOLOGICAL SOCIETY

BY


N. AMBACHE R. V. COXON D. J. C. CUNNINGHAM
M. DE B. DALY P. M. DANIEL S. P. DATTA
G. GORDON CATHERINE HEBB O. F. HUTTER
R. J. LINDEN P. A. MERTON W. J. O'CONNOR
W. A. H. RUSHTON D. H. SMYTH
G. S. BRINDLEY (*Secretary*) B. KATZ (*Chairman*)



September 1961

Volume 158, No. 1

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W. 1

American Branch: 32 East 57th Street, New York 22, N.Y.

*Price 30s. net*

(*U.S.A.* $5.00)

**Notice to Contributors.** Papers submitted for publication should be sent to:

## THE EDITORS OF THE JOURNAL OF PHYSIOLOGY

*Physiology Department, The Medical College of St Bartholomew's Hospital,*

*Charterhouse Square, London, E.C.* 1

Contributors should follow the instructions in '**Suggestions to Authors**' with regard to the preparation of papers, and should study carefully the format of papers in recent numbers of the Journal. The latest edition of this pamphlet appeared in Vol. 150, p. 1. Offprints may be obtained from **The Cambridge University Press, 200 Euston Road, London, N.W. 1.** Price 2*s.* in paper cover, post free.

Papers submitted for publication should be accompanied by a declaration in the following terms: The attached paper entitled ..... has not been, and is not intended to be published anywhere except in the Journal of Physiology, and we/I agree that, if and when it is accepted by the Editors for publication, in consideration for such acceptance the entire copyright in this paper shall pass thereupon to the Physiological Society.

Requests for permission to reproduce any part of papers published in the Journal of Physiology should be addressed to the Secretary of the Editorial Board at the Physiological Laboratory, Cambridge. The Editorial Board will normally grant all requests from authors to reproduce material from their own papers. Permission will not be given to third parties except with the consent of the authors concerned, but if the Editorial Board receives no reply within a reasonable period of writing to the authors at their last address notified, consent will be assumed.

When a paper is submitted by several authors in collaboration their names will be printed in alphabetical order.

The date of receipt printed at the head of each paper is normally the date on which the typescript is first received by the Editors, but if a paper has to be extensively altered after submission the date may at the discretion of the Editors be changed to that on which an acceptable draft is received.

Authors are reminded that the acceptance of papers may be prevented by their being faulty in English composition. It is also essential that papers should be written as concisely as possible, without undue repetition of material in text, tables and figures. Details under the heading 'Methods' will appear in small print unless the author particularly wishes otherwise. Papers concerned with methods only are not acceptable, unless the methods involve new principles and are shown to be of proved value and interest to physiologists. Long theoretical discussions are in general unsuitable.

In an exceptional case the Editors will be prepared to accept a coloured illustration if they consider it to be necessary for conveying the scientific content of the paper. Half the cost of reproduction will be charged to the author.

Authors are requested to verify that the wording of references to unpublished work, and of acknowledgements of help or criticism, is approved by the persons concerned.

The Editors cannot accept responsibility for damage to or loss of papers submitted to them. Contributors are advised to keep copies of their typescripts and illustrations.

Proofs should be corrected and returned promptly. Unless otherwise directed, proofs are sent to the author whose name is first alphabetically, at the address from which the paper originates. If the author(s) wish the proofs to be sent to any other person, or to any other address, instructions should be given

**Abstract.** Authors should send with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Journal of Physiology* but will be scrutinized by the Editors before being passed for publication in *International Abstracts*. It should contain a brief outline of the results and definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet, and should be headed as follows: Title of paper; name(s) of author(s); *J. Physiol., Lond.*, followed by space for year, volume and page reference; abbreviated postal address including country of origin. For example:

A quantitative description of membrane current and its application to conduction and excitation in nerve. A. L. Hodgkin and A. F. Huxley. *J. Physiol., Lond.*, 1952, **117**, 500–544 (Physiol. Lab., Univ. Camb., Cambridge, England).—Text of abstract follows straight on.

The short title *J. Physiol., Lond.*, is used in the abstract to avoid confusion with *J. Physiol., Paris*, but should not be employed in the reference lists of papers submitted to the Journal.

# THE BRITISH JOURNAL OF NUTRITION

**PUBLISHED FOR THE NUTRITION SOCIETY**

*Vol. 15, No. 3 Shortened Versions of Titles 1961 35s. net*

Release of vitamin A from liver. By W. A. McGillivray.

Conversion of carotenoids into vitamin A. By W. A. McGillivray.

Cassava diets for pigs in Nigeria. By V. A. Oyenuga.

Nutritive value of traditionally ground cereals in Southern Rhodesia. By W. R. Carr.

The metabolism of nitrogen, calcium and phosphorus in undernourished children. 7. Effect of replacing rice in diet by pearl millet. By P. P. Kurien, M. Swaminathan and V. Subrahmanyan.

Magnesium in ruminant nutrition. 3. By A. C. Field.

Intraruminal infusions of fatty acids, and yield and composition of milk. By J. A. F. Rook and C. C. Balch.

Saliva secretion and its relation to feeding in cattle. 1. By C. B. Bailey and C. C. Balch.

Saliva secretion and its relation to feeding in cattle. 2. By C. B. Bailey and C. C. Balch.

Availability of lysine in groundnut biscuits used in the treatment of kwashiorkor. By K. J. Carpenter and Beryl E. March.

Illness, calcium intake and rate of skeletal maturation in children. By Audrey Cahn and A. F. Roche.

Nutritive value of lucerne-leaf proteins. By B. K. Sur.

Effect of tocopherol, vitamin A, selenium and anti-oxidants on ubiquinone in the rat. By A. T. Diplock, E. E. Edwin, J. Bunyan and J. Green.

Saliva secretion and its relation to feeding in cattle. 3. By C. B. Bailey.

Analytical differences between muscles of cattle. By R. A. Lawrie.

*Combined Subscription for The British Journal of Nutrition (4 issues) and*

*The Proceedings of the Nutrition Society (2 issues) is £7 per annum.*

**Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1**



# JOURNAL OF ENDOCRINOLOGY

**CONTENTS FOR PART 3 OF VOL. 22, JUNE 1961. 27s. 6d.**

Meites, J. and Hopkins, T. F. Mechanism of action of oxytocin in retarding mammary involution: study in hypophysectomized rats.

Stárka, L. and Malíková, Jitka. A simple method for the separation of pregnanetriols by spread-layer chromatography.

van Zyl, A. The influence of iodide and elemental iodine on protein-bound iodine and serum lipids in the baboon (*Papio ursinus*).

van Zyl, A., Gillman, J. and Gilbert, C. Serum protein-bound iodine and serum lipid changes in the thyroidectomized, hypophysectomized, castrated and adrenalectomized baboon (*Papio ursinus*).

Howell, J. S. and Mandl, Anita M. The mammary glands of senile nulliparous and multiparous rats.

Mandl, Anita M. Cyclical changes in the vaginal smears of senile nulliparous and multiparous rats.

Lehrman, D. S. and Brody, P. Does prolactin induce incubation behaviour in the ring dove?

Lorraine, J. A. and Mackay, Marie A. A comparison of various extraction methods for urinary gonadotrophins with special reference to yields.

Grillo, T. Adesanya I. The ontogeny of insulin secretion in the chick embryo.

Jones, H. E. H., Waynforth, H. B. and Pope, G. S. The effect of miroestrol on vaginal cornification, pituitary function and pregnancy in the rat.

Jones, H. E. H. and Pope, G. S. A method for the isolation of miroestrol from *Pueraria mirifica*.

Cowie, A. T. and Tindal, J. S. Hypophysectomy of the goat.

Debackere, M., Peeters, G. and Tuyttens, N. Reflex release of an oxytocic hormone by stimulation of genital organs in male and female sheep studied by a cross-circulation technique.

Proceedings of the Society for Endocrinology.

*Subscriptions (90s. per volume of 4 parts) should be sent to the*

**Cambridge University Press, 200 Euston Road, London, N.W. 1**

**PHYSIOLOGY OF THE SALIVARY GLANDS**

**A. S. V. Burgen,** M.D., M.R.C.P.
*Professor of Physiology, McGill University*

**N. G. Emmelin,** M.D.
*Professor of Physiology, University of Lund, Sweden*

No. 8 of the Monographs of the Physiological Society:

An account of the latest work on salivary gland function by two eminent scientists from Canada and Sweden. It includes chapters on Theories of Secretion; Metabolism; Physiological Role of Saliva; and Comparative Physiology of the Salivary Glands.

35*s.* net

**EDWARD ARNOLD (Publishers) LTD.**
**41 Maddox Street, London, W.1**

*Particulars of the*

***JOURNALS***

*Published by the Cambridge University Press, may be had from*

THE MANAGER
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD
LONDON, N.W. 1

**INTERNATIONAL ABSTRACTS OF BIOLOGICAL SCIENCES**

**AIMS AND SCOPE**

INTERNATIONAL ABSTRACTS OF BIOLOGICAL SCIENCES attempts to cover the more important papers in experimental biology, approaching the subject from the fundamental point of view, with emphasis on

ANATOMY
BIOCHEMISTRY
BIOPHYSICS
GENETICS
IMMUNOLOGY
PHARMACOLOGY
AND
PHYSIOLOGY

BOTANY and ZOOLOGY are treated as experimental subjects, and little that is purely descriptive is included. Abstracts of ANIMAL BEHAVIOUR are included.

Biochemical and physiological aspects of MICROBIOLOGY and PATHOLOGY are covered. ODONTOLOGY, DENTAL PATHOLOGY and DENTAL ANATOMY are also included.

**JOURNALS COVERED**

A list of the journals (currently over 400) which are regularly scrutinized by the abstractors is given in the first number of each volume. In the majority of cases an indicative abstract of the paper is prepared, but in a few cases—for instance, if the paper is a preliminary communication or the subject matter is border-line—a title only is given.

The total number of abstracts published in a year exceeds 24,000. Monthly issues contain about 2000 abstracts each. Three issues and index of subjects and authors constitute a quarterly volume.

SUBSCRIPTION RATES

*Price per volume (post free)* £10 ($28.00) *Printed on one side of the page* £10. 10*s.* ($29.50)
*Annual subscription (4 volumes)* £35 ($98.00)

**PERGAMON PRESS** Oxford - London - New York - Paris
HEADINGTON HILL HALL, OXFORD 4 & 5 FITZROY SQUARE, LONDON W1
122 EAST 55TH STREET, NEW YORK 22, N.Y.

# THE JOURNAL OF PHYSIOLOGY

★ The subscription price, for orders placed in advance of publication, for vols. 158, 159 (1961) is 80*s.* per volume of 3 parts post free ($13.50 in the U.S.A.).

In normal circumstances, five volumes will appear in each year.

Orders may be placed with your Bookseller, or sent direct to the

**Cambridge University Press,**
**Bentley House, 200 Euston Road, London, N.W. 1**

★ Also available from the **Cambridge University Press**

Back numbers from vol. 61 (1926) onwards.

Volumes 61–115, price £4. 10*s.* per volume.

Volumes 116–120, price £4 per volume.

Volumes 121–130, price £3. 15*s.* per volume.

Volumes 131–157, price £4. 10*s.* per volume.

Single issues and complete sets can be supplied.

Author and subject indexes to the Journal of Physiology, prepared by Dr Grace Eggleton.

Vols. 61–100. 30*s.* net.

Vols. 101–115. 30*s.* net.

★ Available from **Wm. Dawson & Sons, Ltd.**
**16 West Street, Farnham, Surrey, England**

Back numbers, vols. 1–25. Price £6 per volume.
Members' rates on application.

Ready shortly, vols. 20–60 bound, £8 per volume.

Some back issues and volumes are available from Volumes 1–60, particulars on request.

Author index of the Journal of Physiology, vols. 1–60. 25*s.* net.

Subject index of the Journal of Physiology, vols. 1–60, prepared by Dr J. G. Priestley. 15*s.* net.

History of the Physiological Society during its first fifty years 1876–1926. By Sir Edward Sharpey-Schafer, F.R.S. 15*s.* net.

*J. Physiol.* (1961), **158**, *pp.* 1–14
*With 5 text-figures*
*Printed in Great Britain*



# PHYSICAL CHARACTERISTICS OF THE CHEST AND LUNGS AND THE WORK OF BREATHING IN DIFFERENT MAMMALIAN SPECIES

By M. L. CROSFILL* and J. G. WIDDICOMBE

*From the Department of Physiology, Medical College of St Bartholomew's Hospital, London, E.C.* 1

(*Received* 9 *January* 1961)

The most obvious difference in the patterns of breathing in different mammalian species is that small animals breathe faster than large ones. While this may be related to the greater metabolic rate of small animals (in terms of body size) this can be only part of the explanation; for the normal breathing rate of a mouse is greater than a man can maintain for any period of time. Although the factors determining alveolar ventilation rate have received considerable attention, the mechanisms which control the frequency and depth of breathing are less clear. Otis, Fenn & Rahn (1950) have shown that for any given alveolar ventilation rate there is an optimal frequency of breathing, and that if a frequency greater or less than this is adopted (with corresponding adjustments of the tidal volume to maintain the same alveolar ventilation) the work of moving the lungs is greater. Christie (1953) has shown that the 'optimal rate' is adopted in several physiological and pathological conditions, and McIlroy, Marshall & Christie (1954) have suggested that the pulmonary stretch receptors (mediating the Hering–Breuer inflation reflex) are responsible. Mead (1960), on the other hand, has presented results for guinea-pigs and human beings indicating that breathing rate and depth are adjusted to keep the average force of the respiratory muscles minimal, rather than the work performed on the lungs. Whichever view is correct, information on the control of respiratory rate and depth needs to be extended, and the controlling mechanisms worked out.

We have assessed the mechanical properties of the lungs in a number of different species of experimental mammals to see if the values are consistent with the interspecific differences in breathing pattern. Apart from applying the results to the optimal rate hypothesis, the results are values for commonly used experimental animals which may be worth recording.

* Present address: Royal Masonic Hospital, London, W. 6.

## METHODS

Animals were anaesthetized with pentobarbitone sodium (Nembutal, Abbott Laboratories, Ltd), 32–64 mg/kg intraperitoneally (intravenously in dogs and rabbits). Tracheal cannulae were inserted. Intra-oesophageal pressure was measured, through a saline-filled catheter, by a capacitance manometer; the catheter was of length and bore appropriate to the species. The frequency response varied with the catheter, but was never less than 75 c/s (mice). Intra-oesophageal pressure was measured simultaneously with intrapleural pressure in cats and rabbits, and was usually a few cm $H_2O$ above the latter, presumably owing to oesophageal tone and the weight of the thoracic viscera. However, the variations in pressure at the two sites agreed well in quiet breathing; intra-oesophageal pressures were therefore used to calculate transpulmonary pressure, and were expressed relative to the pressures at the end of expiration as zero. During lung inflations intratracheal pressure was measured by an air-filled capacitance manometer attached to the tracheal cannula.

Air flow was measured with gauze-screen pneumotachographs. These were similar to, but proportionally smaller than, that described for man by Lilley (1950). The pressure differential was measured by a sensitive capacitance manometer. For mice it was impracticable to make a pneumotachograph, and flow was assessed by the pressure drop across a constriction in the tracheal tube; this gave a non-linear calibration.

Lung volume changes were usually measured by integrating the flow record; this was done either electrically or planimetrically, the latter being more accurate in slow-breathing animals owing to the time constant of the electrical integrating circuit (40–80 sec). In some experiments volume changes were recorded by connecting the pneumotachograph to a large air reservoir and measuring the pressure changes. The reservoir sizes were adjusted to the animals' tidal volumes, to make sure that the pressure swings were less than 1 cm $H_2O$.

*Compliance.* This was measured under static and dynamic conditions. For the former the lungs were inflated with various volumes of air, while measuring intratracheal and intra-oesophageal pressures. From these a transpulmonary pressure–inflation volume curve was drawn, with the origin corresponding to values at the expiratory level. As Bernstein (1957) has shown for the rabbit, the curves are slightly non-linear, but for comparison between different species linearity was assumed (assessed graphically) and the slope was taken as the compliance value. Dynamic compliance was calculated during eupnoea (see below). Static and dynamic compliance estimates agreed well, and the latter only are given here.

*Non-elastic resistance.* This was calculated from the pressure–flow relations during eupnoea. Although it was possible to calculate coefficients of non-elastic resistance for streamline and turbulent air flow this was not usually done. First, because the turbulent resistance was usually far smaller than that for streamline flow and more difficult to estimate accurately; secondly, because the coefficients (as measured in these experiments) would include a factor due to tissue viscosity; and thirdly because the use of two coefficients makes comparison between different species more complicated. Plots of air flow in eupnoea against the component of transpulmonary pressure causing flow were approximately linear, justifying the use of a linear coefficient only. In man the tissue viscous resistance is a small proportion (about 10 %) of total non-elastic resistance (Marshall & Dubois, 1956), and if this is true for the animals used in these experiments then the results mainly refer to airway resistance.

Non-elastic resistance ($R$) was calculated from synchronous measurements of air flow ($\dot{V}$) and intra-oesophageal pressure difference ($P$) from that at the expiratory level. These have the relationship

$$P = \frac{V}{C} + R\dot{V},$$

where $V$ is the increase in lung volume above expiratory level and $C$ is the compliance of the lungs. The equation is usually solved by measuring $P$ when $\dot{V} = 0$ (at the peak of inflation) and then calculating $C$ from a knowledge of $V$. The relationship between $P - V/C$ and $\dot{V}$ is

then plotted graphically to give $R$. The accuracy of this method depends on measuring $P$ at a point of zero air flow, which is difficult to do precisely in rapidly breathing animals, while in slowly breathing animals $P - V/C$ is small and difficult to estimate accurately. Greater accuracy was obtained by rearranging the equation:

$$\frac{P}{\dot{V}} = \frac{V}{C\dot{V}} + R.$$

$P$, $V$ and $\dot{V}$ were measured at simultaneous times throughout the respiratory cycle, and $P/\dot{V}$ plotted against $V/\dot{V}$. The resulting line, if straight, cuts the ordinate at $R$, and has slope $1/C$. This method usually gave straight lines, which suggests that ignoring the coefficient of turbulent resistance and the assumption of a linear compliance are both justified. A similar method has been used by Nisell & Dubois (1954).

*Chest-wall compliance.* This was measured simultaneously with static lung compliance (as described above); intra-oesophageal (i.e. trans-chest wall) pressure was plotted against inflation volume. The pressure was measured 1–2 sec after inflation of the lungs during the apnoea caused by the Hering–Breuer reflex. It was assumed that this reflex caused complete relaxation of the respiratory muscles. Expiratory muscle tone sometimes rose considerably during large inflations, but only late during the reflex apnoea.

*Functional residual capacity* (*F.R.C.*). The methods of measuring functional residual capacity in man are not easily applicable to small animals. It was therefore determined by measuring total lung volume and subtracting the volume of the tissues; the latter was found from the lung weight, assuming a specific gravity of 1. The lungs were removed from the chest immediately after clamping the trachea in expiration, and total volume determined by immersion in saline. The results may be too low owing to the absorption of gas under the elastic tension of the lungs, but the error is probably small. In many animals the F.R.C. was estimated immediately on opening the chest by withdrawing all possible gas into a syringe; on replacement of the gas and determination of the F.R.C. as described above, similar but slightly larger values were obtained.

*Dead-space volume.* Conventional methods for measuring dead-space volume are not applicable to small animals, so an estimate was made by taking the dead-space volume as equal to one third of the eupnoeic tidal volume. This extremely approximate method of assessing dead-space volume was only needed to apply the optimal rate equations, and its validity will be discussed later. The values obtained agree reasonably with measured dead-space volumes for the dog (Altman, Gibson & Wang, 1958), cat (Folkow & Pappenheimer, 1955) and guinea-pig (Amdur & Mead, 1958).

*Work of breathing.* This was derived from the formula of Otis *et al.* (1950), modified to exclude the term for work overcoming turbulent resistance.

$$W = \frac{F}{2C}\left(\frac{\dot{V}_A}{F} + V_D\right)^2 + \frac{1}{4}\pi^2 R(\dot{V}_A + FV_D)^2,$$

where $W$ is the rate of work, $F$ is the frequency of breathing, $\dot{V}_A$ is the alveolar ventilation rate and $V_D$ is the dead space volume. $\dot{V}_A$ was calculated from the observed frequency of breathing and tidal volume and estimated dead-space volume. The latter was assumed to be constant over the range of breathing frequencies.

The frequency of breathing at which the work on the lungs was minimal was given by

$$F_{m.w.} = \frac{\sqrt{(1 + 4\pi^2 RC\ \dot{V}_A/V_D)} - 1}{2\pi^2 RC}$$

derived from the work equation above (Otis *et al.* 1950; Mead, 1960). The frequency of breathing at which the average force of the respiratory muscles was minimal was calculated from Mead's (1960) equation

$$F_{m.p.} = \left(\frac{\dot{V}_A}{V_D}\right)^{\frac{1}{2}} (2\pi RC)^{-\frac{1}{2}}.$$

Results are given for four animals of each species. In some species, especially mouse, cat and rabbit, many more than four individuals were studied, but if the results were incomplete the entire experiment was excluded from the analysis. No experiments were done on man, so for comparison with the other mammals values have been obtained from the literature.

## RESULTS AND DISCUSSION

The scanty literature on mammalian breathing patterns has been reviewed by Altman *et al.* (1958). Most measurements reported there show wide scatter in any species and, with exceptions which will be noted, our results fall within these variations. Table 1 gives values for a number of respiratory measurements for the eight species.

*Lung specific gravities.* Lung densities fall into two groups, those of the larger species (monkey, cat, dog and man) being 0·20–0·35 g/ml., and for the smaller species (mouse, rat, guinea-pig and rabbit) 0·40–0·51 g/ml. The calculated specific gravity of human lung (0·35 g/ml.) may be too high, since the method of measuring lung weight in man from late post-mortem specimens probably includes more blood than our method for animals involving exsanguination. Spitzka (1904) gives 534 g as the mean weight of lungs removed immediately after death from six electrocuted criminals; he described the lungs as 'bloodless'.

Generally speaking the larger the animals of different species the greater the alveolar diameter (Macklin & Hartroft, 1940; Aumonier, Crosfill & Widdicombe, 1958). The cat is an exception. We therefore expected to find a decrease in specific gravity with body size. A clearer distinction, however, is between the two groups of species mentioned above. This might be correlated with a very thin serous pleura in the four smaller species, and a thicker fibrous pleura in the four larger, if the proportion of stromal tissue follows the type of pleura.

*Lung compliance.* The intrapleural-pressure swings to produce tidal volume changes do not show great interspecific differences, neither do the minute volumes per kilogram body weight, with the exception of man, lower than average, and rat and mouse, higher. Correlation with body surface areas, had they been known, might have given closer values.

Table 2 gives lung compliances in absolute values, and in terms of lung weight and of total lung volume at functional residual capacity level. Expressed as compliance per unit total lung volume at functional residual capacity level (Fig. 1), there is little interspecific difference, except that the rabbit has a high compliance and man a low value. Expressed per gram of lung weight (Table 2) compliance increases with specific body size, with the exceptions of dog and especially man which have relatively stiff lungs.

Van Liew (1954) found a lung compliance of 90 ml./cm $H_2O$ for a dog, and since it weighed 20 kg this value agrees reasonably with ours. With

TABLE 1. Values for various respiratory measurements in eight species

| | Body weight (kg) | Lung weight (g) | Functional residual capacity (ml.) | Specific gravity (g/ml.) | Tidal volume (ml.) | Tidal pressure swing (cm $H_2O$) | Minute volume (l./min) | Minute volume per kg body wt. (l./min/kg) | Frequency (breaths/min) | Mean alveolar diameter ($\mu$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Mouse | 0·032 | 0·20 | 0·29 | 0·42 | 0·18 | 3·6 | 0·021 | 0·72 | 109 | 38·69 |
| | 0·027–0·038 | 0·17–0·23 | 0·17–0·49 | 0·31–0·53 | 0·09–0·38 | 2·0–5·5 | 0·009–0·046 | 0·24–1·72 | 97–123 | |
| Rat | 0·25 | 1·6 | 1·55 | 0·51 | 1·55 | 4·3 | 0·16 | 0·61 | 97 | 59·13 |
| | 0·19–0·32 | 1·3–1·95 | 1·2–1·9 | 0·47–0·58 | 1·03–2·13 | 3·1–4·9 | 0·09–0·27 | 0·46–0·85 | 84–126 | |
| Guinea-pig | 0·69 | 3·2 | 4·75 | 0·40 | 3·7 | 3·2 | 0·13 | 0·21 | 42 | 83·43 |
| | 0·43–1·05 | 2·4–4·1 | 4·1–5·1 | 0·37–0·43 | 2·3–5·3 | 2·1–5·1 | 0·08–0·19 | 0·12–0·38 | 16–67 | |
| Rabbit | 2·4 | 9·1 | 11·3 | 0·43 | 15·8 | 2·9 | 0·62 | 0·25 | 39 | 93·97 |
| | 2·05–3·0 | 7·0–10·5 | 7·2–15·8 | 0·37–0·59 | 11·5–24·4 | 2·3–4·2 | 0·37–0·89 | 0·18–0·33 | 32–53 | |
| Monkey | 2·45 | 22·3 | 87·5 | 0·20 | 20 | 1·7 | 0·70 | 0·32 | 33 | 89·06 |
| | 1·8–3·05 | 13·5–30 | 61–115 | 0·14–0·29 | 9–29 | 1·3–2·3 | 0·26–1·34 | 0·10–0·76 | 27–47 | |
| Cat | 3·7 | 20 | 66 | 0·23 | 34 | 2·6 | 0·96 | 0·31 | 30 | 133·19 |
| | 2·3–5·7 | 13–26 | 41·5–92 | 0·21–0·25 | 20–42 | 1·9–3·7 | 0·86–1·09 | 0·18–0·47 | 24–42 | |
| Dog | 12·6 | 82 | 252 | 0·23 | 144 | 3·8 | 3·1 | 0·23 | 21 | 74·05 |
| | 10·0–15·5 | 44–105 | 206–340 | 0·18–0·33 | 122–176 | 2·9–4·8 | 0·8–5·5 | 0·08–0·35 | 6–31 | |
| Man | 70 | 1065 | 2000 | 0·35 | 400 | 4·5 | 6·4 | 0·091 | 16 | 166·11 |

Means and ranges are given. Values for man are for conscious supine adult subjects at rest; lung weights are post-mortem (Altman *et al.* 1958). Alveolar diameters are from Macklin & Hartroft (1940).

TABLE 2. Values for lung and chest wall compliance, and lung resistance

| Species | Lung compliance (ml./cm $H_2O$) | | | Chest-wall compliance (ml./cm $H_2O$) | | | Lung resistance (cm $H_2O$/l./sec) | | | Compliance ratio chest/lung | Time constant (sec) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Absolute | per g lung | per ml. lung | Absolute | per g lung | per ml. lung | Absolute | per g lung | per ml. lung | | |
| Mouse | 0·049 | 0·26 | 0·11 | 0·33 | 1·68 | 0·68 | 480 | 89 | 395 | 8·0 | 0·03 |
| | 0·025–0·068 | 0·11–0·40 | 0·04–0·15 | 0·16–0·41 | 0·86–2·35 | 0·45–0·89 | 267–1310 | 50·5–303 | 95·7–940 | 3·1–16·4 | 0·014–0·05 |
| Rat | 0·39 | 0·23 | 0·12 | 1·47 | 1·07 | 0·55 | 90 | 145 | 277 | 4·65 | 0·035 |
| | 0·22–0·52 | 0·16–0·32 | 0·09–0·19 | 1·22–2·11 | 0·82–1·28 | 0·43–0·70 | 71–119 | 113–196 | 197–418 | 3·75–5·56 | 0·024–0·044 |
| Guinea-pig | 1·26 | 0·38 | 0·15 | 3·66 | 1·00 | 0·46 | 59 | 191 | 480 | 3·25 | 0·075 |
| | 0·76–2·33 | 0·31–0·57 | 0·12–0·25 | 2·07–4·83 | 0·87–1·63 | 0·32–0·62 | 81–148 | 148–267 | 337–675 | 1·94–5·16 | 0·057–0·088 |
| Rabbit | 6·0 | 0·64 | 0·28 | 9·4 | 1·04 | 0·47 | 25 | 222 | 522 | 1·81 | 0·15 |
| | 3·5–10·8 | 0·44–1·04 | 0·19–0·41 | 8·2–10·6 | 0·94–1·20 | 0·40–0·60 | 15·3–42 | 159–445 | 400–732 | 0·95–2·43 | 0·087–0·193 |
| Monkey | 12·3 | 0·66 | 0·12 | 7·3 | 0·41 | 0·069 | 8·6 | 145 | 1028 | 0·56 | 0·10 |
| | 7·1–20·2 | 0·34–1·05 | 0·08–0·22 | 4·4–14·4 | 0·20–1·06 | 0·037–0·15 | 4·4–14·2 | 60–318 | 416–1550 | 0·47–0·71 | 0·080–0·133 |
| Cat | 13·4 | 0·70 | 0·16 | 13·4 | 0·70 | 0·16 | 11·5 | 196 | 865 | 1·02 | 0·14 |
| | 9·9–17·4 | 0·62–0·76 | 0·13–0·18 | 7·2–17·3 | 0·48–1·06 | 0·10–0·25 | 8·8–12·1 | 152–267 | 670–1060 | 0·66–1·39 | 0·12–0·18 |
| Dog | 40 | 0·50 | 0·12 | 32 | 0·47 | 0·098 | 1·3 | 102 | 480 | 0·81 | 0·055 |
| | 27–61 | 0·37–0·62 | 0·09–0·19 | 21–49 | 0·24–0·54 | 0·063–0·15 | 0·75–2·03 | 73–181 | 235–696 | 0·64–0·93 | 0·045–0·067 |
| Man | 200 | 0·19 | 0·065 | 208 | 0·20 | 0·068 | 0·90 | 960 | 2780 | 1·04 | 0·18 |

Means and ranges are given. Values per ml. of lung refer to total volume including lung tissue (at expiratory level). Resistance values exclude the resistance of the tracheal cannulae and apparatus. Human values are from Altman *et al.* (1958), except the chest-wall compliance figures, which are from Butler & Smith (1957). The identity of mean lung and chest-wall compliances for the cat is not an error in tabulation.

nine cats Nisell & Dubois (1954) obtained values of 3·9–10 ml./cm $H_2O$, but the weights of their cats were considerably less than ours, so again the results agree reasonably. For 200 guinea-pigs Amdur & Mead (1958) give a mean value of 0·20 ml./cm $H_2O$, and even allowing for a weight difference this is a considerably lower compliance than our results. The lung resistance of their guinea-pigs was also much higher. Kautsky (1957)

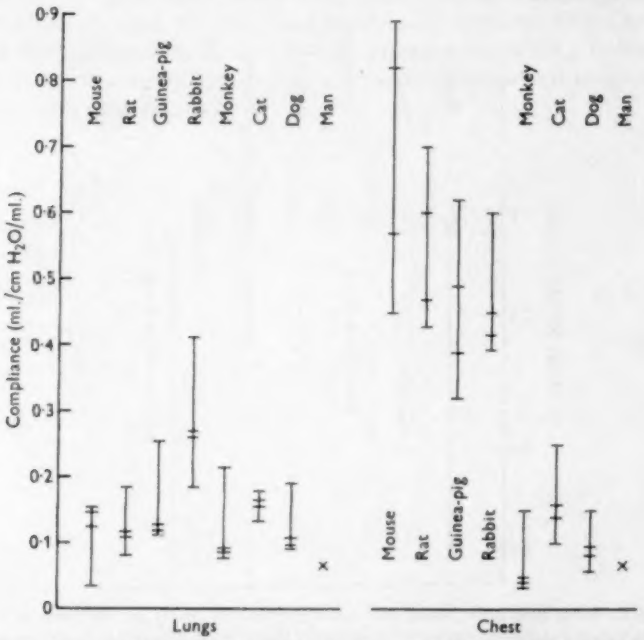



Fig. 1. Lung and chest-wall compliances per millilitre total lung volume at functional residual capacity level. Each horizontal line represents the measurement from one individual animal.

measured compliances of cat, rabbit, guinea-pig, rat and mouse; for the rabbit and guinea-pig he found lower compliances than ours, but the other species give good agreement.

*Chest-wall compliance.* The species fell into two groups; the four smaller had a high chest-wall compliance per unit lung volume, the four larger species a low compliance (Table 2, Fig. 1). Calculated as a ratio of lung compliance (Table 2) there was an inverse relationship between chest-wall compliance and body size. The measurement of chest-wall compliance was based on the assumption that the muscles of breathing were completely relaxed when the Hering–Breuer inflation reflex was active, a view which

was supported by observation of the animals but not tested by electromyographic recording. Since the functional residual capacity of an animal is related both to lung compliance and to chest-wall compliance, the low functional residual capacities and high lung specific gravities found in mouse, rat, guinea-pig and rabbit may well be related to the high chest-wall compliance, the relaxation position of the thorax corresponding to a relatively small volume.

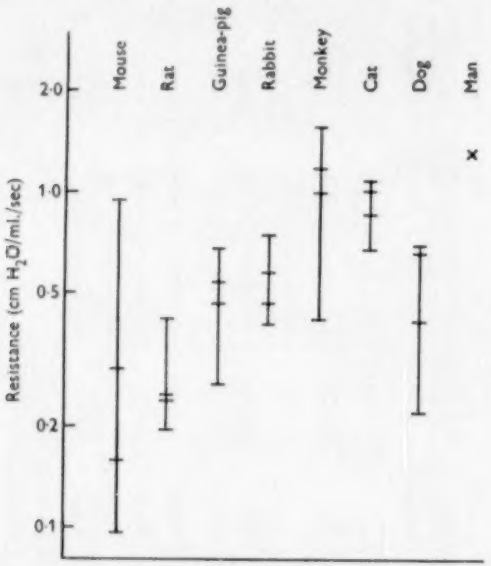



Fig. 2. Lung resistance (tissue and lower respiratory tract) per millilitre of total lung volume at functional residual capacity level. Each horizontal line represents the measurement from one individual animal. Ordinate on log scale.

*Lung resistance.* This tended to increase with specific body weight (Table 2, Fig. 2), although the dog had a relatively low resistance. The great scatter in results for the mouse probably indicates the difficulty in making accurate measurements of resistance in this species. Resistance for man has been taken as half the published figure for total resistance of the lungs and respiratory tract, since about 50% of the airway resistance is thought to lie in the upper respiratory tract (Radford, 1957) which was excluded in the experimental animals. Amdur & Mead (1958) have shown that for guinea-pigs the upper respiratory tract accounts for a mean of 45% of the total resistance of air flow of the respiratory system, and one of us (J. G. W.) found that in cats and rabbits with closed mouths the

resistances of upper and lower respiratory tracts were about equal. (Two cats had upper airway resistances of 9·4 and 8·7 cm $H_2O$/l./sec with pulmonary resistances of 12·5 and 13·2 cm $H_2O$/l./sec respectively; three rabbits had upper airway resistances of 13·3, 17·4 and 28·0 cm $H_2O$/l./sec with corresponding pulmonary resistances of 14·1, 15·2 and 25 cm $H_2O$/l./sec. The upper airway resistances were very easily increased by changing the position of the animals' heads and necks.) If the resistances in pulmonary units of equal size were the same, large animals would have in addition the resistance to flow in the air passages conducting to the pulmonary units; this would increase the total resistance per unit lung volume compared with small animals.

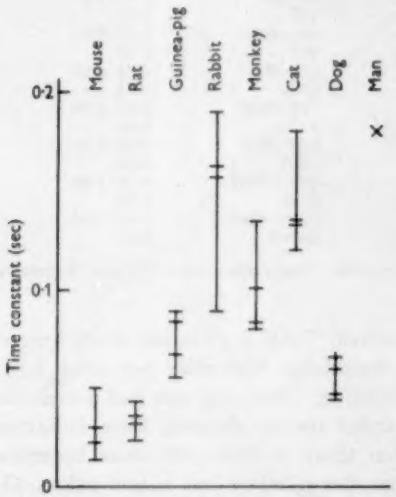



Fig. 3. Time constants (compliance multiplied by resistance). Each horizontal line represents the measurement from one individual animal.

*Time constants.* Compliance multiplied by resistance gives the time constant (analogous to the time constant of a resistance–capacitance electrical circuit) of the lungs. Animals with short time constants could have rapid alterations in lung volume with little phase shift. The time constants run roughly parallel to the breathing rates of the species (Fig. 3) although the dog has a particularly short time constant. It is our impression that, of the species examined, the dog most commonly breathes rapidly and shallowly to lose body heat; a short time constant of the lungs would therefore be of advantage. The time constants given in Table 2 and Fig. 3 do not include allowance for the upper airway resistance and the

mechanical properties of the chest wall. Nisell & Dubois (1954) found mean time constants of 0·163 and 0·093 sec for two groups each of nine cats with cannulated tracheae. Brody (1954) working with tracheotomized dogs gave figures of 0·148 (8 dogs) and 0·079 sec (15 dogs). Other measurements of time constants in man and experimental animals have included the resistance of the upper airways.

TABLE 3. Values for the work of breathing

| Species | Work of breathing (g. cm/min) | | |
|---|---|---|---|
| | Absolute | per g body weight | per unit ventilation |
| Mouse | 66·5 | 4·25 | 2540 |
| | 15–194 | 0·47–7·2 | 1560–4220 |
| Rat | 482 | 1·90 | 2940 |
| | 251–1006 | 1·18–3·37 | 2160–3740 |
| Guinea-pig | 272 | 0·52 | 2090 |
| | 179–390 | 0·28–0·91 | 1560–2880 |
| Rabbit | 1502 | 0·62 | 2370 |
| | 798–2500 | 0·39–1·09 | 1960–3280 |
| Monkey | 817 | 0·31 | 1157 |
| | 223–1418 | 0·09–0·57 | 861–1450 |
| Cat | 1857 | 0·63 | 1900 |
| | 1321–2883 | 0·31–1·26 | 1505–2650 |
| Dog | 6720 | 0·55 | 2350 |
| | 2233–9485 | 0·32–0·78 | 1695–2775 |
| Man | 30,000 | 0·43 | 4700 |

Means and ranges are given. Unit ventilation is 1 l./min. Values for man are from Altman *et al.* (1958).

*The work of breathing.* Table 3 gives the work (in g.cm/min) of moving the lungs during breathing, the work per gram body weight, and the work per unit ventilation. Mice and rats had a high rate of work per unit body weight, the other species showing little difference; when expressed per unit ventilation there is little difference between the experimental species, except that the monkey has a low value. The values for man, taken from Otis *et al.* (1950), include work to overcome airway resistance in the upper respiratory tract and they are high compared with the experimental animals. If the minute volumes bear the same relation to metabolic rates in the different species, then about the same proportions of the animals' metabolic rates are devoted to moving the lungs.

*Optimal rates of breathing.* Figure 4 shows the measured rates of breathing plotted against the rates for minimal work performed on the lungs, calculated as described in Methods. Here the resistance values include the resistances of the tracheal cannulae, since this in effect takes the place of an upper airway resistance, and McIlroy, Eldridge, Thomas & Christie (1956) have indicated that the mechanism controlling breathing rate and depth is sensitive to external resistances. The correspondence between observed and optimal rates is striking, and was unexpected in

view of the many possible sources of discrepancy. The animals were anaesthetized, which might be expected to inhibit the mechanisms adjusting breathing rates to the optimal; the upper respiratory tract was eliminated and replaced by tracheal cannulae; chest-wall compliance and viscance were not included in the calculation; resistance was expressed as a linear relationship between air-flow and resistive pressure, ignoring

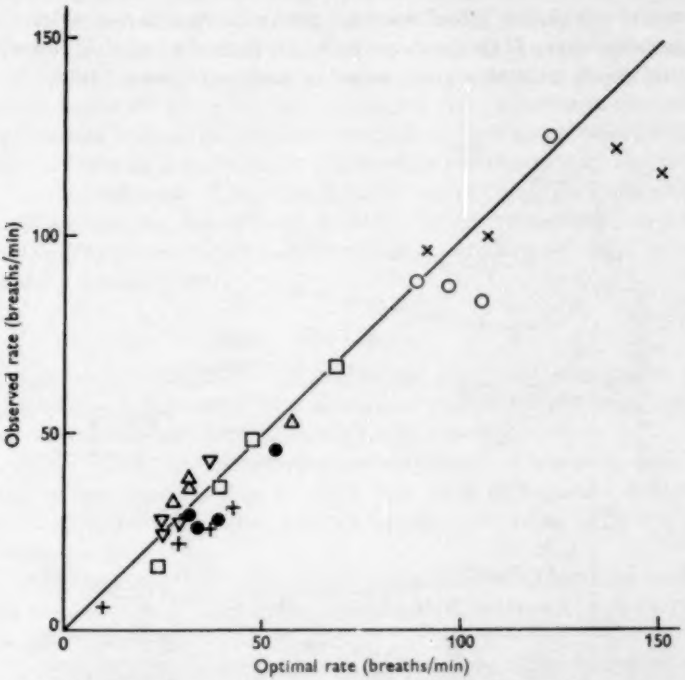



Fig. 4. Measured rates of breathing plotted against the calculated optimal rates for four animals of each species. Mouse ×, rat ○, guinea-pig □, rabbit △, monkey ●, cat ▽, dog +.

possible turbulence in the air passages; and dead-space volume, a knowledge of which is essential for an accurate solution of the optimal rate equation, was crudely estimated and assumed to be constant in any individual animal.

Typical optimal rate curves for individuals of different species are shown in Fig. 5. The optimal rates are shown by the arrows, and the rates which correspond to a 10% increase in work from the minimum are shown by the transecting lines. It might be better to refer to the 'efficient range' of

breathing rates rather than the 'optimal rate', at least for the smaller animals; for example, the mouse whose work of breathing is shown could vary its frequency of respiration from 81 to 190/min with an extra expenditure of energy of only 10% at these limits. For man the total energy expenditure of the respiratory muscles at rest is only about 5% of the metabolic rate, and our results suggest a similar or smaller proportion in the experimental animals, so a considerable deviation from the optimal rate would not greatly affect the total metabolic rate at rest. Within such a wide range there is ample scope for other factors to modify respiratory rate and depth without a great waste of energy (Dejours, 1960).

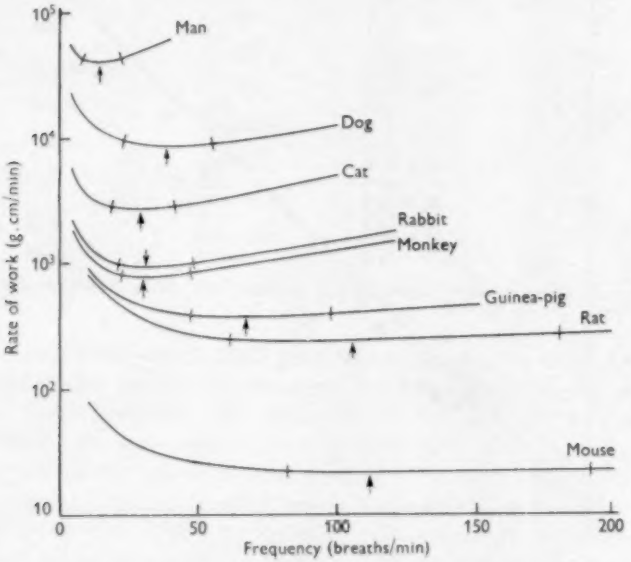



Fig. 5. Curves relating the work of breathing (ordinate, log scale) to the possible frequencies of breathing (abscissa) which give constant alveolar ventilations. One animal of each species is represented; the values for man are from Otis *et al.* (1950). The arrows indicate the optimal rate of each individual, the transecting lines show the points where work would be 10% greater than the minimum.

If Mead's (1960) equation (for calculating the breathing frequency at which the average respiratory muscle force is minimal) is applied to our results, then the optimal frequencies are very greatly in excess of the observed frequencies (mean 96% in excess, range 48–295%). It is not fully justifiable to apply either optimal-rate equation to our results, since both require knowledge of the compliance and resistance of the total

respiratory system, including extrapulmonary structures. Both formulae depend on the two factors, time constant and alveolar ventilation: dead-space ratio. Unless the time constant of the chest wall differs greatly from that of the lungs, omission of the mechanical properties of the chest will not greatly affect the calculated optimal rates. Replacement of the upper airway resistance by a tracheal cannula would influence the optimal rates only in so far as the animals cannot adjust the breathing pattern to allow for variation in resistance; the extent to which they can do this is disputed (McIlroy *et al.* 1956; Mead, 1960). The resistance of the tracheal cannulae was of the same order as that of the upper respiratory tracts in those species in which the latter has been measured. It is inherent in the optimal rate equations that as the alveolar–ventilation: dead-space ratio increases, the optimal rate for minimal force will increase less slowly than the optimal rate for minimal work. The animals in our experiments had low alveolar ventilations due to anaesthesia, and at higher ventilation rates their breathing frequencies might have fitted better the hypothesis of minimal respiratory muscle force.

## SUMMARY

1. Pulmonary compliance, resistance and chest-wall compliance have been measured in four individual animals of each of the following species: mouse, rat, guinea-pig, rabbit, monkey, cat and dog.

2. When pulmonary compliance was expressed in terms of unit lung volume at the expiratory level, there was little interspecific difference, except that rabbit lungs were more compliant, and human lungs less, than the other species.

3. Lung resistance (per unit lung volume) tended to increase with the size of the animals of each species investigated, although dogs had relatively low resistances.

4. Mice, rats, guinea-pigs and rabbits had very high chest-wall compliances and low functional residual capacities (both per unit lung volume), compared with animals in the other four species.

5. The frequencies of breathing mechanically most economical (optimal rates) were calculated for the individuals, and corresponded quite closely to the observed rates of breathing.

6. Calculation of the mechanical work of breathing of each animal showed that the frequency of breathing could vary considerably for constant alveolar ventilation, with little increase in work; this was especially true for the smaller animals.

Some of the apparatus used in these experiments was bought from the Central Research Fund, University of London.

## REFERENCES


ALTMAN, P. L., GIBSON, J. F. & WANG, C. C. (1958). *Handbook of Respiration*. Saunders: Philadelphia.

AMDUR, M. O. & MEAD, J. (1958). Mechanics of respiration in unanesthetized guinea pigs. *Amer. J. Physiol.* **192**, 364–368.

AUMONIER, F. J., CROSFILL, M. L. & WIDDICOMBE, J. G. (1958). The relationship between lung compliance and histology in different species. *J. Physiol.* **141**, 20*P*.

BERNSTEIN, L. (1957). The elastic pressure–volume curves of the lungs and thorax of the living rabbit. *J. Physiol.* **138**, 473–487.

BRODY, A. W. (1954). Mechanical compliance and resistance of the lung–thorax calculated from the flow recorded during passive expiration. *Amer. J. Physiol.* **178**, 189–196.

BUTLER, J. & SMITH, B. H. (1957). Pressure–volume relationships of the chest in the completely relaxed anaesthetized patient. *Clin. Sci.* **16**, 125–146.

CHRISTIE, R. V. (1953). Dyspnoea in relation to the visco-elastic properties of the lungs. *Proc. R. Soc. Med.* **46**, 381–386.

DEJOURS, P. (1960). Selection of a ventilatory regime, and the theory of minimum work. *Physiologist*, **3**, 49.

FOLKOW, B. & PAPPENHEIMER, J. R. (1955). Components of the respiratory dead space and their variation with pressure breathing and with bronchoactive drugs. *J. appl. Physiol.* **8**, 102–110.

KAUTSKY, E. (1957). Vergleichende Untersuchungen über den elastischen Lungenwiderstand. *Helv. physiol. acta*, **15**, 358–360.

LILLEY, J. C. (1950). Flowmeter for recording respiratory flow of human subjects. In *Methods in Medical Research*, **2**, 113–121, ed. COMROE, J. H. Chicago: Year Book.

MCILROY, N. B., ELDRIDGE, F. L., THOMAS, J. P. & CHRISTIE, R. V. (1956). The effect of added elastic and non-elastic resistances on the pattern of breathing in normal subjects. *Clin. Sci.* **15**, 337–344.

MCILROY, M. B., MARSHALL, R. & CHRISTIE, R. V. (1954). The work of breathing in normal subjects. *Clin. Sci.* **13**, 127–136.

MACKLIN, C. C. & HARTROFT, W. S. (1940). The size of pulmonic alveoli based on measurements of their outline in 25 $\mu$ microsections of human and common laboratory animals' lungs fixed in the state of expansion. *Extramural Report* C.P. 35. Canada Subcommittee on Physiological Aspects of Chemical Warfare.

MARSHALL, R. & DUBOIS, A. B. (1956). The measurement of the viscous resistance of the lung tissues in normal man. *Clin. Sci.* **15**, 161–170.

MEAD, J. (1960). Control of respiratory frequency. *J. appl. Physiol.* **15**, 325–336.

NISELL, O. I. & DUBOIS, A. B. (1954). Relationship between compliance and FRC of the lungs in cats, and measurement of resistance of breathing. *Amer. J. Physiol.* **178**, 206–210.

OTIS, A. B., FENN, W. O. & RAHN, H. (1950). Mechanics of breathing in man. *J. appl. Physiol.* **2**, 592–607.

RADFORD, E. P. (1957). Recent studies of mechanical properties in mammalian lungs. In *Tissue Elasticity*, pp. 177–189. American Physiological Society.

SPITZKA, A. (1904). A note on the true weight of human lungs. *Amer. J. Anat.* **3**, v.

VAN LIEW, H. D. (1954). Contribution of vagus nerves to pressure–volume characteristics of chest and lungs in dogs. *Amer. J. Physiol.* **177**, 161–163.

*J. Physiol.* (1961), **158**, *pp.* 15–38
*With* 12 *text-figures*
*Printed in Great Britain*



# EXCITATION OF RECEPTORS IN THE PAD OF THE CAT BY SINGLE AND DOUBLE MECHANICAL PULSES

BY CHRISTINE J. ARMETT* AND R. W. HUNSPERGER†
*From the Department of Physiology, University College London*

(*Received* 11 *January* 1961)

Information about the strength and position of one or more points of mechanical contact with the skin may be transmitted in a number of nerve fibres acting as an organized group. The coding of such information in a group of receptor fibres from the cat's pad has been investigated by C. J. Armett, J. A. B. Gray, R. W. Hunsperger and S. Lal (unpublished), who have also investigated the way in which this code is modified at the first synaptic junction. A single stimulus, having a particular position and strength, sets up in the primary fibres a pattern of activity which is, within limits, uniquely related to that stimulus. The mechanism which determines this pattern is naturally of interest, because an understanding of it would enable the patterns of activity set up by more complicated combinations of stimuli to be predicted. Basic points in this problem are the size of the receptive fields and the amount of overlap between them. Such receptive fields might be determined by a branching of the receptor nerve fibre to cover the area; on the other hand a single receptor in the centre of the field would be all that would be required if the stimulus set up a mechanical wave that travelled across the pad. The experiments described in this paper are concerned with this problem, and the results indicate that a mechanical wave does travel across the pad as a result of each stimulus and that this wave is important in determining the pattern of activity that results in the primary fibres.

The first section of the results deals with experiments in which single stimuli were used to excite single receptor units. The second section deals with the effects of a mechanical pulse on the excitability of the units to a second pulse.

## METHODS

The experiments were performed on cats anaesthetized with chloralose, 0·05 g/kg, and urethane, 0·5 g/kg. The lumbar cord was exposed to give access to the dorsal roots, and the animal was then mounted rigidly in a frame (cf. Fernandez de Molina & Gray, 1957); steel

* Present address: Department of Pharmacology, Albert Einstein College of Medicine, Morris Park Avenue and Eastchester Road, Bronx, 61, N.Y., U.S.A.

† Present address: Physiological Institute, University of Zurich, Zurich, Rämistr. 69, Switzerland.

needles were passed ventral to the bodies of lumbar vertebrae, but dorsal to the transverse processes, and were then fastened to the frame. A hind foot was mounted, pad upwards, on a brass plate attached to the frame; the foot was held by a stitch, and the whole, including the pad, stabilized by embedding it up to the level of the pad surface in paraffin wax. The medial plantar nerve was exposed, laid over a pair of electrodes and then embedded in low-melting-point paraffin wax. The lateral plantar nerve was cut.

*Mechanical stimulation.* Mechanical pulses were derived from Rochelle salt crystals (Gray & Malcolm, 1950); in these experiments larger crystals 2·5 $cm^2$ were used and they were damped with silicone grease instead of oil. Two such crystals were needed and each was mounted on a manipulator, which was graduated in steps of 10 $\mu$ in the three planes at right angles to each other. The glass styli, by which the displacements of these crystals were transmitted to the pad, had tip diameters of *ca.* 0·5 mm. When a stylus was lowered to the surface of the pad, the point of contact could be seen by the electrical output from the crystal. The position of contact could be established in this way, correct to a few tens of micra, and the resting position was usually adjusted to be 0·3–0·75 mm deep to this position of contact.

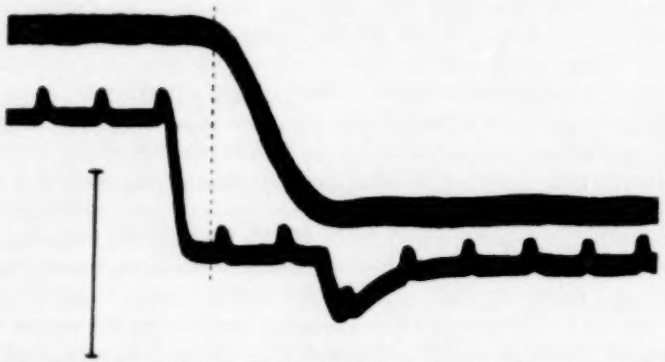


Fig. 1. Displacement of the moving corner of the crystal (top) after the application of a rectangular voltage pulse followed by a voltage pulse having an exponential decay (bottom). Calibration 50 V, time marker, 100 $\mu$sec. Note: the displacement of the crystal starts with a delay of about 80 $\mu$sec.

The crystals had a sensitivity of approximately 0·4 $\mu$/V and the largest voltage used was 56 V. When a pulse of less than 20 V was applied to a crystal, the resulting displacement, if critically damped, was 90% complete in about 200 $\mu$sec. Imperfections in the voltage source introduced greater lags when pulses greater than 20 V were used. In a few experiments adequately damped pulses having shorter rise times were required; these were obtained by driving the crystals from a source having a sufficiently low output impedance at all pulse amplitudes, and allowing oscillation to occur; these oscillations were then counteracted by adding a brief pulse of suitable size, wave form and timing (Fig. 1).

*Recording.* Nerve impulses were recorded from fine filaments of dorsal-root or occasionally of medial-plantar nerve, with platinum electrodes under liquid paraffin and an amplifier system having a high input impedance and a frequency response which was adjusted to give the optimum conditions for determining the presence or absence of an impulse. The activity of a single receptor fibre excited by mechanical stimulation of the pad could be detected amongst considerable activities from other sources by using a superimposing technique; the stimulus sequence was repeated continuously at 10/sec, but the time base was switched on

and off by electrical means so that groups of 5 or 10 sweeps were superimposed on each photograph. The general activity could be reduced by cutting unwanted nerves in the leg.

*Recording mechanical events.* Displacements were recorded by a modification of the optical system described by Gray & Malcolm (1951). The optical part of this apparatus was used to record the displacements of a nylon bristle running in a guide (Fig. 2). The upper end of the nylon bristle held a flag, which interrupted the light beam of the optical system. The

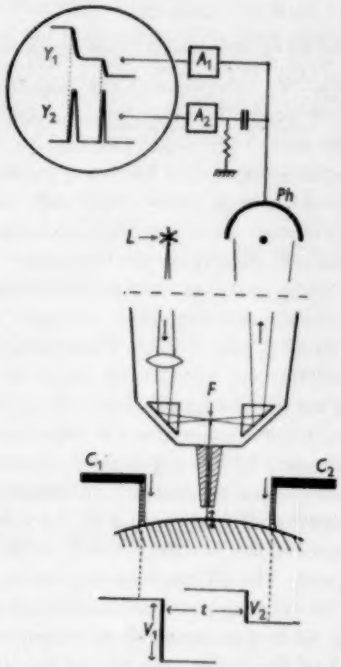



Fig. 2. Diagram of the method used for recording the displacement and the velocity of the surface movements of the pad. *L* light source, *F* flag on the recording stylus interrupting a focused light beam, *Ph* multiplier photocell. In this example a voltage pulse, $V_1$, is applied to crystal $C_1$; it causes a displacement of which the amplitude is recorded via $A_1$ and $Y_1$ and of which the velocity is recorded via $A_2$ and $Y_2$. After an interval $t$ a voltage pulse $V_2$ is applied to crystal $C_2$ and the displacements due to this appear as the second deflexions on $Y_1$ and $Y_2$.

mechanical impedance of the system was kept as small as possible, the mass was 5 mg, the viscous resistance was small but was not satisfactorily measured and there was no elastic component. The restoring force provided by the weight of the system was small, and it was necessary, if movements were to be followed, to stick the bristle to the pad or crystal by means of collodion. When this recording device was attached to the moving corner of a crystal it was found that the photocell output was linearly related to the voltage input to the crystal. It may therefore be concluded that under these conditions, and over this working range, the output of the instrument was linearly related to the displacement of the nylon bristle. However, it was not possible to eliminate a tendency for the bristle to stick when

the forces were small, as was the case when small displacements of the pad surface were recorded; the method could not therefore be used for certain quantitative measurements. Displacements as small as 1 $\mu$ could be recorded. The velocity of the displacement was recorded by differentiating the output of the displacement recorder (Fig. 2). This was done by means of a circuit having a time constant of 20 $\mu$sec.

## RESULTS

### *Responses of receptor units to single mechanical pulses*

*On-and-off responses.* The receptors of the pad which could be excited by the means employed were, like other phasic receptors, excited both by the movement into the pad at the beginning and by the restoration movement at the end of a mechanical pulse having a duration of at least several milliseconds (for refs. see Gray, 1959); normally one impulse only was initiated by each movement when the displacement amplitude was 20 $\mu$ or less. Movements in both directions are thus able to excite. It is known that in the Pacinian corpuscle movements in both directions set up negative running receptor potentials and that these receptor potentials are able to sum in the usual way (Gray & Sato, 1953). The experiments described in this paper indicate that mechanical waves may occur in the pad and that the phases of such waves may add and subtract. It might be expected, then, that the effects of the two movements could sum under certain conditions. That they do so is indicated by the experiment illustrated in Fig. 3. In this experiment the threshold to a restoration movement was lower than that to the inward movement at the beginning of the pulse. It was possible to distinguish the responses to the two movements from each other by altering the duration of the pulse; the off-response moved in time with the end of the stimulus, while the on-response remained fixed in time. The line with the full circles (Fig. 3) is the strength–duration curve of the response occurring at the end of the pulse; the points for pulses of 3 and 5 msec duration indicate the threshold to the restoration movement alone; the amplitudes of pulses with durations between 0·7 and 2 msec did not need to be so large to produce a response, though it is certain that the impulse was initiated by the end of the pulse; the thresholds with the two shortest pulses were higher, and with these it was not possible to distinguish the effects of the two movements. It was possible to obtain a response to the inward movement of the pulse by increasing the amplitude of any of the pulses which had a duration longer than 0·7 msec; the threshold value for a response to this movement is shown by the open circles in Fig. 3, and it can be seen that it was unaffected by the duration of the stimulus. An increase in the excitability was thus observed when both movements were in a position to contribute, but not when the beginning of the pulse was in itself sufficient to excite.

This is not the preparation in which to study the meaning of the large amplitudes required for excitation by short pulses. It can, however, be said that the displacement of the pad normal to its surface at a distance of 1·5 mm from the stimulator was only slightly reduced when the pulse was as short as 0·3 msec; the record of displacement against time had a definite plateau when the pulse had a duration of 0·5 msec.

*The relation between the parameters of the pulse and excitation.* For excitation to occur in these receptors, as in other phasic receptors which are mechanically excitable, a minimum displacement and a minimum velocity (or critical slope) had to be exceeded. This is well illustrated by plotting,

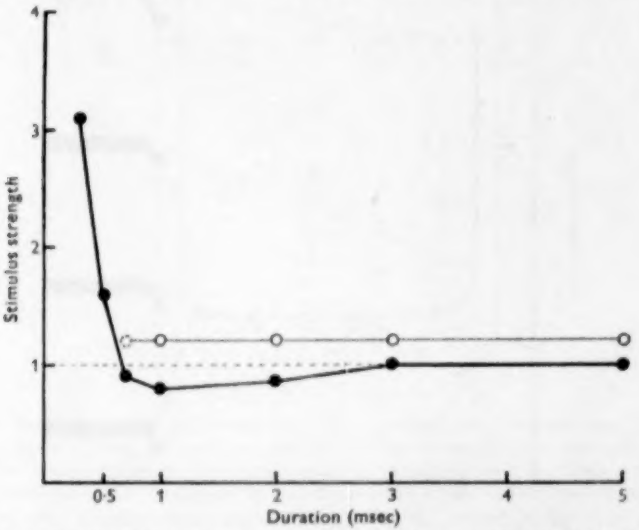



Fig. 3. Plot of the amplitude of displacement for threshold against pulse duration. Ordinate, stimulus strength in multiples of the threshold of a restoration movement. Abscissa, pulse duration. ●, lowest threshold; ○, threshold of the response locked to the beginning of the pulse. With pulses of 2 msec duration or less no stimulus excited more than one impulse; with longer pulses responses to both displacement and restoration could occur on the same occasion.

for threshold, the amplitude of displacement of a long pulse against the velocity of the initial movement. The results of two such experiments are illustrated in Fig. 4. The full curve was obtained when using pulses shaped by the method illustrated in Fig. 1; the displacement of the stimulating crystal was recorded throughout and four of the pulses are illustrated. The interrupted curve is taken from another experiment, in which the special technique for shaping the pulse was not used. Both curves are similar to those found with other receptors which can be excited mechanically (Gray

& Matthews, 1951; Gray & Malcolm, 1951) and show that when the velocity was above the critical value, threshold was determined by a critical level of the amplitude of the displacement, but was virtually unaffected by changes of velocity. In contrast, when the amplitude was above a certain value, threshold was determined by the velocity and was little affected by changes of amplitude. The critical slopes in three experiments were 1·3, 1·6 and 1·8 minimum thresholds/msec; the mean value for critical velocity in absolute units was 6·5 $\mu$/sec.

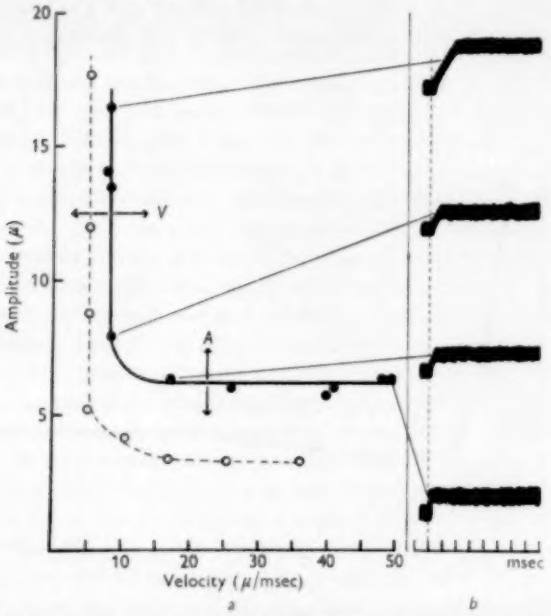



Fig. 4. Relation between amplitude and velocity of displacement for the excitation of receptor units. (*a*) ●—● curve found with pulses obtained by the method illustrated in Fig. 1. ○---○ curve obtained in another experiment with a single rectangular voltage pulse applied to crystal. Arrows: *A*, region in which amplitude determines excitation; *V*, region in which velocity determines excitation. (*b*) Records of the displacements used at threshold for the four different points indicated. Time marker, msec.

*Receptive fields.* It was possible to excite a single receptor unit over quite large areas. For example, with 14 units tested with brief (*ca.* 0·6 msec) mechanical pulses, having amplitudes up to about 20 $\mu$, the areas from which responses could be obtained varied from 25 to 120 mm$^2$. Of these, the larger extended over the whole of the large pad; in one instance it was possible to excite the unit from one of the small pads as well as from the

whole of the large one. It must be emphasized that these areas are for one particular size of stimulus. A receptive field could be an area over which branches of the receptor neurone spread, but defined by this test method it could also be the area over which there is sufficient mechanical spread to excite a single receptor; or, of course, it could be a mixture of both. It will emerge later in this paper that mechanical spread is probably the major factor, and if that is so the area from which responses can be obtained will be determined by the size of the test pulse.

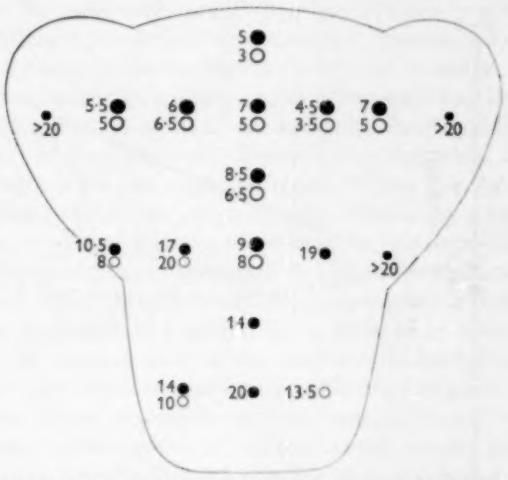



Fig. 5. Receptive field of a single receptor unit on the large pad. Separate threshold measurements for the displacement (●) and the restoration (○) of a mechanical pulse of 10 msec duration. The numbers indicate the amplitudes of the movements in $\mu$; both thresholds were tested at all points. When a point is indicated by one symbol only, it means that a response could only be obtained to that type of stimulus. When neither direction excited, a small filled circle marked > 20 is used. The medium-sized circles indicate thresholds > 10 $\mu$ and the largest circles values < 10 $\mu$.

In order to understand the signalling of information by primary and higher receptor neurones, it is important to consider certain factors about a receptive field defined in this way: these are the amplitude of displacement at threshold, the velocity of displacement at threshold, and the latency of initiation of an impulse at different points in the field. In all preparations there was an area of maximum sensitivity, and as the distance from this area increased the minimum displacement required to excite increased. Figure 5 illustrates a field which was tested with a long pulse in order that both on- and off-thresholds might be recorded. The numbers beside the filled circles indicate the amplitudes of the initial movements at

threshold, and those beside the open circles indicate the thresholds for the restoration movements. The pattern is not a simple one, but this like other experiments indicates that the excitability was much reduced at the periphery of the field. A corollary to this was found in examining the size of eleven such receptive fields mapped out with short (*ca.* 0·6 msec) pulses; the thresholds in the most sensitive area varied from 1·5 to 16·5 $\mu$ and those whose thresholds at the centre were lowest had the largest fields when explored with a 20 $\mu$ deformation (correlation coefficient = −0·69). Since

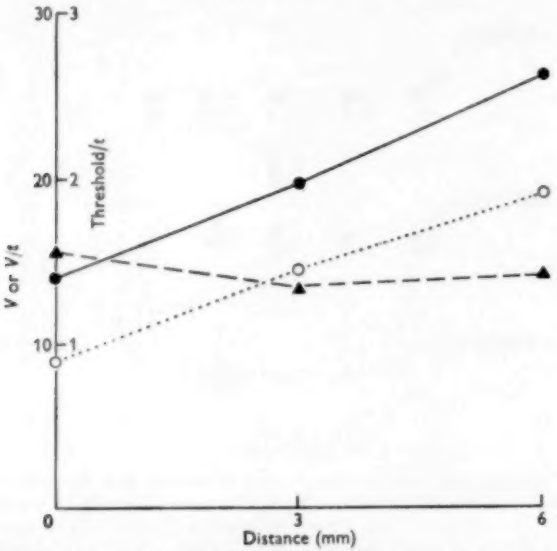



Fig. 6. Relation between threshold and distance. Ordinate, velocity or amplitude; abscissa, distance on the pad from the position of lowest threshold. ● Velocity at threshold expressed as (volts to crystal)/msec; ○ amplitude at threshold expressed as volts to crystal; ▲ velocity at threshold expressed as thresholds/msec., i.e. critical slope.

stimuli of the same size could excite from greater distances when the receptor was more excitable, it looks as though excitation at a distance was determined by mechanical spread.

The critical velocity required for excitation also increased with distance from the most excitable region. Figure 6 illustrates the change, with distance from the most sensitive region, in the size of the critical velocity (filled circles) and the critical amplitude (open circles) for excitation. The third line presents the same results in another way: the slope is plotted as multiples of threshold amplitude per unit time (i.e. critical slope). This line

is almost horizontal, and indicates that the time in which the minimum amplitude must be reached is constant over the receptive field.

The latency of the initiation of an impulse tended to increase with distance from the most sensitive region; the results were not, however, very consistent. There are two factors which account for this increase. One factor derives from the stimulus-strength–latency relationship (Fig. 7), which was similar to those found for other receptors (e.g. Gray & Malcolm, 1950, 1951); in this figure the ordinate is the increase in the latency above the minimum value. When the latencies were measured at threshold in different parts of the receptive field, fluctuations occurred and this accounts for much of the inconsistency referred to above. If, however, latencies were measured with a large pulse of constant amplitude (e.g. the largest available), then, in terms of the threshold, the stimulus was smaller at the periphery of the field, where threshold was high, than at the centre, where threshold was low. As was to be expected from Fig. 7, the relatively smaller stimulus given in the periphery was followed by a longer latency than the relatively larger pulse in the centre. In one experiment series of stimuli of different amplitude were used at a number of points throughout a receptive field. Each stimulus could then be recorded in multiples of the threshold at that particular position; the differences in latency to a constant stimulus in different parts of the receptive field could in large part, but not entirely, be accounted for by the differences in sensitivity of each point. The other factor responsible for the increase in latency with distance is the time taken for the spread of the mechanical events. An increase in latency due to this factor is illustrated in Fig. 11 (open circles); the reasons for attributing this delay to mechanical causes are given in a later section where this experiment is described.

Observations of the displacement of the pad surface in a direction normal to that surface were made in a number of preparations, using the optical method already described. All records of displacement at all distances from the stimulator were in the same direction, and the amplitude of this displacement decreased with distance from the stimulator; no oscillations were seen. On the average the amplitude of the displacement was reduced to a half at a distance of 2 mm; the rate of rise of the displacement was also reduced by the same factor, the time of rise or fall of the pulse being little altered at any distance. The displacement spread across the pad from the stimulator with a definite velocity, and this was found in three experiments to range from 11 to 13 m/sec, and in one to be 32 m/sec. The velocity inferred from the experiment illustrated in Fig. 11 was approximately 11 m/sec. These observations are consistent with the hypothesis that the changes in the amplitude at threshold, in the velocity at threshold, and in the latency observed in different parts of a receptive field, can be explained

by the spread of a mechanical wave. The distances required for attenuation to a half obtained from these mechanical observations, however, appear to be too small to fit the excitability results. On the other hand, displacement normal to the surface is unlikely to be the mechanical factor directly responsible for the excitation of the receptors.

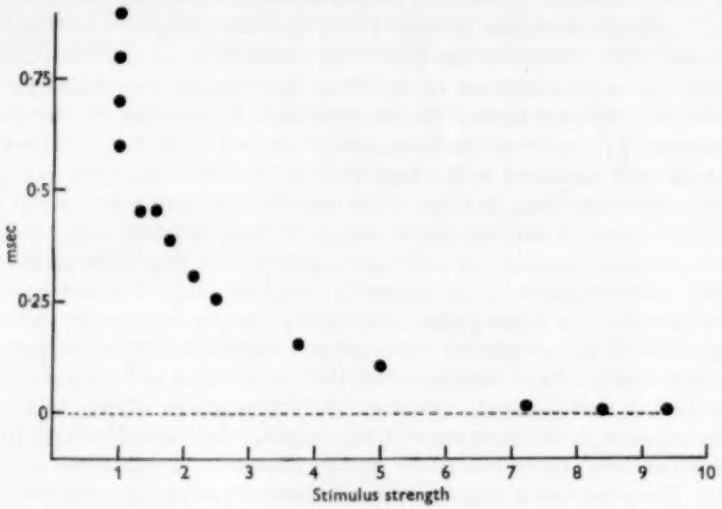



Fig. 7. Relation between stimulus strength and latency of the response. Ordinate, latency difference; abscissa, stimulus strength in multiples of threshold. Duration of the pulse 0·6 msec.

*Recovery of excitability and conduction velocity of the nerve impulse.* In these units a brief mechanical pulse (*ca.* 0·6 msec duration), just large enough to excite on every occasion, could excite again 1·8–2·8 msec (5 units) after the beginning of the first pulse. The largest available stimuli could not excite earlier than about 1 msec after the first pulse.

The conduction velocities of the nerve fibres of those units which could be excited by mechanical stimulation of the pad were measured in a number of experiments. A unit was identified as being excited by mechanical stimulation of the pad and its nerve fibre was excited electrically by means of a 20 $\mu$sec pulse applied to the medial plantar nerve, and the impulse was recorded in the dorsal root. At the end of the experiment the nerve between the two pairs of electrodes was completely excised and measured. In 9 units the mean velocity was 66 m/sec and the range 46–85 m/sec; 6 of the units had velocities between 61 and 67 m/sec.

*Responses of receptor units to pairs of mechanical pulses*

The remainder of the results described in this paper were obtained with two pulses applied to the pad, usually by means of two separate stimulators, which enabled the pulses to be separated both in position and in time. The simple application of the second stimulator to the pad frequently changed the threshold to a pulse from the first stimulator. This occurred even after any necessary correction had been made to the vertical position of the first stimulator. Decreases in excitability were usually seen when the second stimulator was applied within a few millimetres of the first and there was often an increase in excitability when the distance was greater.

*Interaction following two brief threshold pulses.* In a number of experiments a threshold pulse was found to alter the response to a second threshold pulse. With the two stimulators 3–5 mm apart on the large pad each was adjusted to deliver a pulse of 0·6 msec duration, of such an amplitude that it was just able to excite every time when it was applied alone. When both pulses were applied at an interval greater than 2 msec two responses were seen; when the interval was around 1·5 msec only the first pulse excited, because the excitability of the unit had not recovered in time for the second pulse to excite. When the two pulses were delivered simultaneously a single response was also obtained. The surprising result occurred when the interval was around 0·6 msec: at this interval no response of any kind was obtained, even though both pulses were above threshold when given alone. It appeared that a second stimulus was able to prevent the response to one that had already occurred.

*Interactions following the association of a short test pulse with a short subthreshold conditioning pulse.* The qualitative phenomenon just described was investigated by using a conditioning–test technique with short pulses (*ca.* 0·6 msec duration). One pulse, the conditioning pulse, was adjusted to about 80 % of its threshold value and delivered at zero time. The test pulse was derived from the other stimulator 4–9 mm away, and at times within 10 msec before (−) or after (+) the conditioning pulse; at each setting its amplitude was adjusted until the threshold for an impulse in the primary receptor fibre was reached. The result of such an experiment, which is typical of seven others, is shown in Fig. 8. The abscissa of this graph is time before or after the conditioning pulse and the ordinate is given as $(S_\infty - S_t)/S_\infty$, where $S_\infty$ is the stimulus strength at threshold of the test stimulus alone and $S_t$ is the stimulus strength at threshold at time $t$. In words, this function may be described as the fractional lowering of the test threshold resulting from the conditioning stimulus given $t$ msec before. Figure 8 shows two major deflexions, a large decrease in excitability when the test occurred about 0·5 msec before the conditioning and a big increase

in excitability when the pulses were synchronous. There are also smaller deflexions before and after these. Other experiments showed similar patterns, though the relative sizes of the different peaks varied considerably. A common feature, which is small in Fig. 8, was a negative peak at about $+0{\cdot}5-+1{\cdot}0$ msec. The two main peaks of this experiment may be analysed in more detail by considering records of the amplitude and velocity of the displacement of the pad surface, normal to that surface, and at a point midway between the two stimulators (Fig. 2 shows the method). In Fig. 9 there are three sets of records. On top are the voltages applied to the two crystals (these are mixed on one beam in the oscilloscope) and 0·1 msec time marks; the lower rows indicate the velocity (into the pad = upward deflexions) and displacement (into the pad = downward deflexions) recorded from the mid point: in *a* and *b* the velocity is on top, in *c* it is below. These records are typical of a number obtained in this way. Record *a* shows the two pulses separated and giving two distinct displacements and two phases of positive velocity and two of negative; this picture corresponds to an interval when only minor changes in excitability occurred. Record *b* was taken at an interval which was practically identical with the duration of the pulses; under these conditions there is only one, though longer, displacement and the middle two phases of velocity are obliterated. This interval corresponds in all experiments to that at which there was a large decrease in excitability and at which two threshold pulses failed to excite any impulse. This result, like that illustrated in Fig. 3, suggests that each movement, regardless of direction, contributes to the excitation of the receptor unit. If, as in the Pacinian corpuscle, each movement sets up a negative running receptor potential (Gray & Sato, 1953) or if there is addition and subtraction of mechanical waves, a result such as that just described might be expected.

In three of these seven experiments a single brief pulse of 0·6 msec duration set up two impulses; the interval was about 2 msec. In these preparations a double-pulse experiment, such as that illustrated in Fig. 8, gave high positive values of $(S_\infty - S_t)/S_\infty$, not only when the pulses were applied simultaneously, but also when the interval between the pulses was the same as that found between the impulses of the double response. Double responses were exceptional amongst the whole series of experiments considered in this paper.

A conditioning pulse to a small pad has been seen to affect the threshold to a test pulse on the large pad.

*Interactions following the association of a long test displacement with a long subthreshold conditioning displacement.* The experiments with short pulses which have just been described illustrate in a dramatic way the interactions that can follow two mechanical pulses, and they also suggest

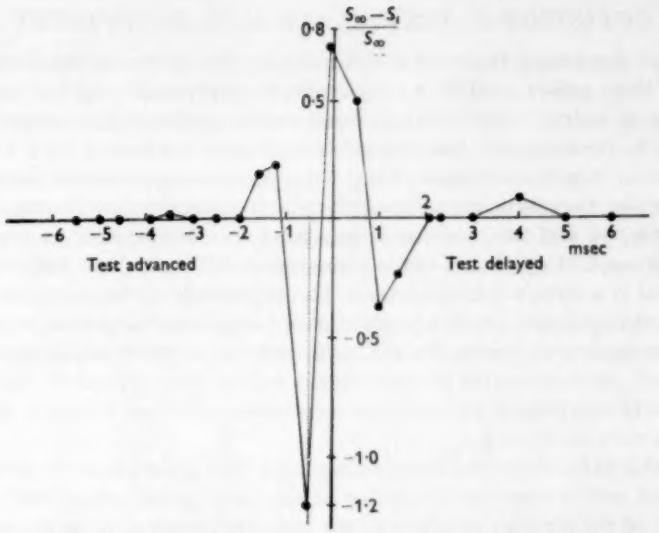



Fig. 8. Contribution of a subthreshold conditioning pulse to excitation by a test pulse. Ordinate, $(S_\infty - S_t)/S_\infty$; abscissa, time of test pulse in relation to conditioning pulse. Distance between the stimulating points 4 mm, pulse duration 0·6 msec. Conditioning threshold 2·9 times test threshold; conditioning used 2·2 times test threshold.

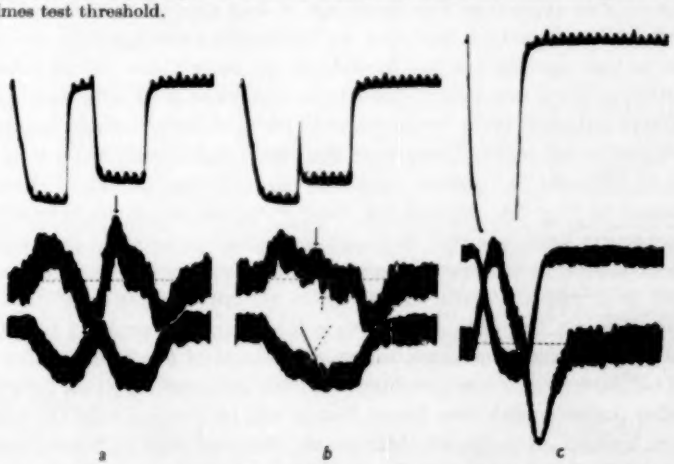



Fig. 9. Mechanical patterns on the surface of the pad following two mechanical pulses of 0·6 msec duration applied at different positions 4·5 mm apart and at different times. Upper row: voltages to both transducers mixed to one beam. Lower rows: displacements and velocities recorded on the pad midway between the stimulating points. Interval between the pulses *a*, 0·9, *b*, 0·6 and *c*, 0 msec. Records with dotted base lines are velocity records (note last pair reversed). The arrows in *a* and *b* indicate corresponding points at the end of the first pulse and at the beginning of the second. For further explanation see text.

that one important factor in this interaction lies in the mechanics of the pad. Short pulses provide a very useful experimental tool, but are not typical of natural stimuli; long displacements probably have more relevance to problems of discrimination and have been used in a further analysis of this phenomenon. Long displacements have another particular advantage; the results in the first section have indicated that the movement into the pad and the movement associated with restoration are separate stimuli, each of which can excite a single impulse if a critical amplitude is reached in a certain minimum time. The beginnings of long displacements of opposite polarity are thus approximately equivalent as stimuli, but they are mechanical opposites. In this section one experiment will be described in detail, as it illustrates all the relevant points. It is typical of two other relatively complete experiments and a number of others in which specific points were confirmed.

In this experiment the test displacement was always a movement into the pad and it was always applied at the same point, which was in the region of the greatest sensitivity; the test displacement, as in the experiment described in the last section, was altered in time relative to the conditioning pulse, and was adjusted in amplitude until an impulse was excited in response to half the number of stimuli. The conditioning displacement was applied in five positions; it was applied 2, 4, 6 and 8 mm from the test stimulator and also, by mixing the voltage into the same crystal as was used for testing, applied at the same point. At all positions conditioning displacements of both polarities were used and their amplitudes were adjusted to be approximately 80% of the threshold amplitude at that particular point. There were thus ten conditioning situations, and a plot of $(S_\infty - S_t)/S_\infty$ against time was made for each. Six of these are illustrated in Fig. 10. In making these runs the intervals between the displacements were adjusted so that the points on the main peaks of the graph do represent the maxima and minima.

With two displacements of the same polarity, applied through one crystal (Fig. 10, top left), there was a large positive peak at zero time; this simply represents a single larger movement of the crystal. The next graph in the top row was obtained with the stimulators 6 mm apart and a similar positive peak was found, but it will be noticed that the peak is delayed by 0·45 msec. In the third graph, obtained with an 8 mm interval, the peak is delayed 0·75 msec. These delays are displayed in a plot against distance as the full circles in Fig. 11. This lag might be due to the time required for a travelling wave to pass from the conditioning stimulator to the test point, which was presumed to be near the receptors. If this were true, then an impulse excited by the conditioning stimulator should be delayed in respect of one excited by the test stimulator. This delay should

also be the time required for the mechanical wave to travel the distance between the two. These increases in the latency of the response at different distances from the most sensitive area were measured, and are indicated by the dotted lines in the graphs of Fig. 10; the values are also plotted against distance as open circles in Fig. 11. It will be seen that the lag in the positive peak of the graphs, and the increase in latency of impulse initiation, are very closely related and it seems reasonable to attribute them to the same cause. That this cause is the travelling of a mechanical wave is supported by the following evidence.

The three graphs in the lower part of Fig. 10 were obtained with conditioning pulses moving in the direction of restoration. It is immediately

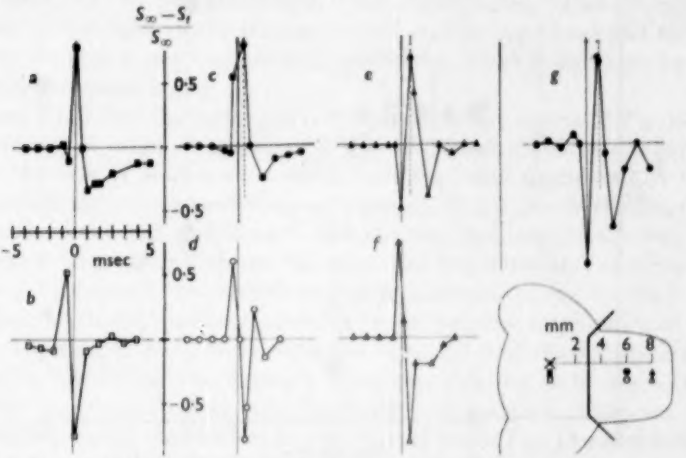



Fig. 10. Contribution of a subthreshold conditioning displacement to the excitation of a receptor unit by a test displacement into pad at different distances. Upper row, conditioning displacement into pad. Lower row, conditioning displacement out of pad. Ordinates, $(S_\infty - S_t)/S_\infty$; abscissae, time interval between the displacements (msec); test advanced negative. The inset in the right-hand bottom corner indicates the distances in mm of the conditioning displacement from the test displacement ( × ). The square bracket drawn on the pad marks the line of removal of the distal part of the pad. For further explanation see text. Conditioning displacements were as follows:

| | Distance from test (mm) | Polarity | (%) of threshold at point used | Size relative to threshold at test point |
|---|---|---|---|---|
| *a* | 0 | In | 85 | 0·85 |
| *b* | 0 | Out | 79 | 1·35 |
| *c* | 6 | In | 82 | 2·1 |
| *d* | 6 | Out | 82 | 4·7 |
| *e* | 8 | In | 86 | 3·4 |
| *f* | 8 | Out | 85 | 4·1 |
| *g* | 6 | In (after cutting) | 82 | 2·3 |

obvious that a negative peak in these records corresponds to the positive peaks in the upper graphs and that this negative peak shows the same lag with increasing distance. This reversal is exactly what would be expected if the interactions were mechanical, but not what one would expect if they were concerned with receptor potential summation and depression. In each instance the negative peak was preceded by a positive peak, and the time between these peaks was constant, within experimental error, at about 0·6 msec. Furthermore, in this experiment the latency from the

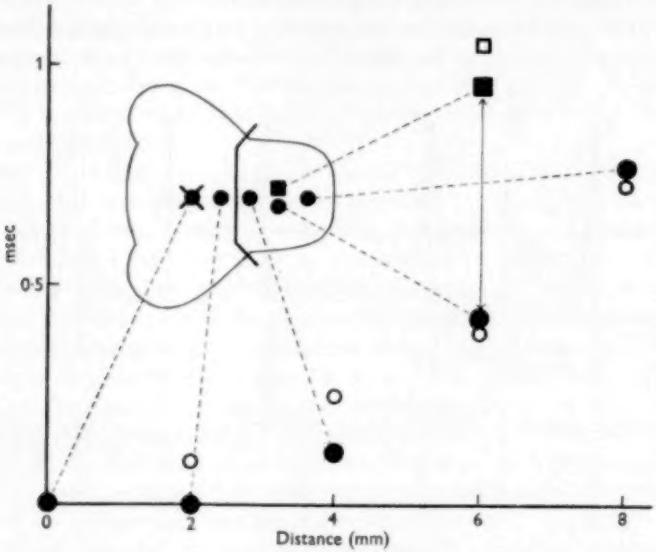



Fig. 11. Lag of peaks of increased excitability (Fig. 10, top row) (●) and increase in latency of a threshold response (○) with distance. Ordinates, lag and increase in latency; abscissa, distance of the conditioning displacement from the test pulse. The inset shows the positions of the points: X test, ● conditioning. The latency measurements were mostly repeatable within 0·1 msec, even though measured at threshold: □, ■, after cutting.

beginning of a stimulating displacement to the initiation of the impulse was 0·6 msec shorter when the displacement was *out* of the pad than when the movement was *into* the pad (the threshold for movements into the pad was, however, lower). These results are all explicable in mechanical terms, if it is assumed that a movement in either direction causes a brief mechanical change with two phases 0·6 msec apart, somewhere in the tissues, and that reversal of the applied movement reverses the order of its phases. It is further postulated that one phase only can excite; in this instance, that occurring first after a restoration movement. This would account for

the extra 0·6 msec latency after an inward displacement and also for the fact that an inward displacement needed to be applied 0·6 msec before an outward moving displacement if their excitatory effects were to sum. On this hypothesis one would expect that if a test displacement into the pad were applied 0·6 msec before a conditioning displacement in the same direction the excitatory phase of the mechanical change following the test stimulus would coincide with and be cancelled by an opposing phase following immediately on the application of second displacement. There is a small negative peak at the correct time when the two displacements were applied through the same stimulator; a corresponding negative peak is, however, much more obvious in results obtained with the two stimulators well apart (Fig. 10, row at the top). In the latter figure the whole graph is shifted to the right on the time scale, and on this hypothesis this shift is due to the spread of a wave at a finite velocity, which is indicated by the slope of the curve in Fig. 11.

Final proof that the major peaks of the interaction curves of Fig. 10 are mechanical in origin is afforded by the right-hand graph in this figure. After the sets of observations which have just been described had been completed, the pad was cut across as indicated by the line in the diagram. The part of the pad peripheral to this cut was then completely removed and stuck back again with paraffin wax. The test stimulator remained in the same place and the conditioning stimulator was placed at the 6 mm position; the latter could still excite at about the same amplitude and was again adjusted to 80% of its threshold. The result of this experiment is shown in the top right-hand graph. It is clear that the peaks are as large or even larger than before; the lag of the positive peak from zero was appreciably longer than before, and the dotted line in Fig. 10 and the point in Fig. 11 show that the latency of the response increased by the same amount. The close correspondence of these points after this large change confirms that the two measurements have the same origin. The increase in these times might well be due to a slowing of the mechanical wave in passing the discontinuity provided by the wax.

Records were made of displacement and velocity of the pad in a direction normal to the surface. These records showed that neither the amplitude nor the rise time of a displacement was affected by the application of a second displacement from another stimulator, either simultaneously or within milliseconds of it; displacements of either polarity from the two stimulators recorded separately at any point were combined algebraically when they were applied together. These recordings did not reveal any oscillations due to mass movements of the stimulator, the pad or foot; the direction and sensitivity of the recording instrument should have revealed any significant mass movements of this kind if they had existed. Further-

more, mass movements could not explain the time delays introduced by distance or by the discontinuity in the cut pad. The displacement records referred to in an earlier section showed that some mechanical change travelled across the pad with a velocity comparable to that found in this experiment, about 11 m/sec. However, the displacements recorded normal to the surface have shown no sign of the postulated brief mechanical change with two phases.

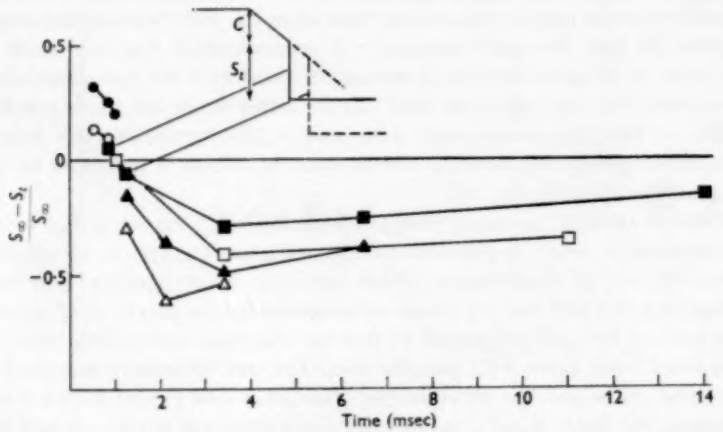



Fig. 12. Excitability changes during velocities below the critical value tested by a rectangular step (see inset); movements into the pad. Ordinate, $(S_\infty - S_t)/S_\infty$; Abscissa, time from start of velocity to test pulse. The slopes (thresholds/msec) were ○ = 0·65; ● = 1·1, △ = 1·4, ▲ = 0·8, □ = 0·55, ■ = 0·4. Critical slope = 1·75 thresholds/msec. $S_t$ = test stimulus. $C$ = conditioning amplitude.

These observations of displacements normal to the surface do not, therefore, provide a full explanation of the results. A few observations were, however, made with a Rochelle-salt crystal which was rigidly attached to a glass stylus. This stylus was applied to the pad. The system could respond to forces normal to the surface and also to forces tangential to the surface. Results obtained with it cannot therefore be analysed. None the less, some records obtained with this system showed deflexions which changed polarity within a millisecond of the beginning of the mechanical change. These records indicate that there are mechanical events which are not revealed by the measurement of displacements normal to the surface, and which may be more closely related to the excitation of the receptors.

It will be noted that the size of the positive peaks in Fig. 10 is approximately the same at all distances. This is to be expected since the size of the conditioning displacement relative to threshold was always the same. The

absolute size of the displacements was, however, greater at the larger distances: values are given in the legend to this figure.

*Low-velocity pulses.* All the experiments described so far, whether with short pulses or long displacements, were done with movements whose velocity was above the critical value. Slower changes may be of importance when considering the discriminative functions of skin. An experiment was therefore done in which the conditioning movement had a velocity which was varied, but always below the critical value (Fig. 12). The test displacement was a high-velocity movement of the same stimulator introduced at some point during the period of the conditioning movement. In this experiment all velocities caused a decrease in the excitability at times greater than 1 msec, and both positive and negative values of $(S_\infty - S_t)/S_\infty$ were greatest with the highest velocities. The rather prolonged, but not very profound, negative change was found in a proportion of results obtained with rectangular displacements, both short and long, and when this preparation was conditioned with a rectangular instead of a sloping displacement.

## DISCUSSION

The purpose of this paper has been to consider how various stimulus patterns affect the excitability of certain receptor units which can be excited by mechanical stimulation of the cat's pad. It is clear that mechanical factors play a major part in determining the response of a unit.

In the experiment illustrated in Figs. 10 and 11 the amplitude of a displacement required to excite was approximately the same even after the part of the pad to which it was applied had been cut out and stuck back. A displacement on this inactive region was also able to modify, as effectively as it could before the area was cut out, the excitability of a unit tested by another stimulus. These facts show that mechanical events can spread across the pad and have effects on receptors at a distance. There is evidence that there is a mechanical wave which spreads across the pad at a definite velocity. This has been indicated in several ways: the latency of initiation of an impulse increased as the distance from the most sensitive area of the pad increased; the peaks of the interaction curves (Fig. 10) were delayed as the conditioning stimulus was removed further from the most sensitive area on which the test stimulus was applied. These two delays in impulse initiation and in all phases of interaction were, within the limits of error, identical at all distances, and even after the pad had been cut out and replaced. They can be accounted for by the time taken for a mechanical wave to travel the distance; the calculated velocity of this postulated wave was similar to the velocity with which a displacement of the pad surface, normal to that surface, was found to spread.

A mechanical wave spreading through the pad would be expected to become attenuated with distance. The amplitude required for threshold increased towards the periphery of receptive fields. The linear dimensions of receptive fields were negatively correlated with the absolute value of threshold in the most sensitive region. The velocity required for threshold also increased towards the periphery when recorded in absolute units, but was unchanged when allowance was made for the change in the amplitude required for threshold. These results all suggest attenuation of the amplitude of a mechanical wave, but little or no change in its time course. Displacements normal to the pad surface were found to decline with distance from the point of application, and the time course of these displacements remained unchanged.

A spreading mechanical wave will have a particular time course and a particular relationship to the applied pulse. Is there then a pattern that will fit the results presented in this paper? The units under discussion are phasic; like other phasic receptors, e.g. in frog's skin (Gray & Malcolm, 1951; Maruhashi, Mizuguchi & Tasaki, 1952) and the Pacinian corpuscle (Gray & Malcolm, 1950), these units respond with a single impulse or very few impulses to any movement regardless of direction. The velocity–amplitude curve for threshold found in the pad is very similar to those found for receptors in frog's skin (Gray & Malcolm, 1951) and for Pacinian corpuscles (Gray & Matthews, 1951). The adaptation of the receptors in frog's skin can be modified by mechanical means (Loewenstein, 1956) and the brief responses to movement and the shape of the velocity–amplitude curve of the Pacinian corpuscle have been explained in mechanical terms by Hubbard (1958) as a result of his investigations into the mechanics of the capsular structure. By analogy one might expect that movements both into and out of the pad might give rise to brief mechanical changes whose amplitude is dependent on both the amplitude and the velocity of the applied displacement.

A mechanical explanation is required for the interactions between two displacements. That these interactions are mechanical is shown by the cutting of the pad and by the fact that reversal of the conditioning displacement reversed the interaction curve; receptor potentials and other receptor processes would not be expected to reverse (for summary, see Gray 1959). The main peaks on the interaction curves, including their polarities, intervals and delays and the on-and-off responses, can be explained if it is postulated that movements into and out of the pad both give rise to a brief mechanical change having two phases of opposite polarity, whose order is reversed by reversing the polarity of the applied pulse; if it is assumed that only one of these phases is able to excite the receptors the results can be explained by the mechanical interference of

these phases. To avoid again describing results here explanations of the details in terms of this hypothesis have been given where the results are described.

There is no direct evidence of the mechanical process postulated. Displacements normal to the skin surface are certainly correlated with some of the properties required, but they are different in other respects. These differences do not, however, invalidate the hypothesis. For example, as the stimulus moves away from the point of lowest threshold the displacement is found to become attenuated more rapidly than does the threshold. This might be due to the receptor unit studied having receptors not only at the point of minimum threshold, but spread over some little area, so that certain receptors may remain close to the stimulus even when it is some distance from the centre of the receptive field. An alternative explanation of these differences in attenuation and of other discrepancies might be that the mechanical change that is important is not that recorded by the method used; observations made with a Rochelle-salt crystal suggest that there are other mechanical changes. A mechanical process which might possibly be more closely associated with the excitation of the receptors is a compression wave travelling in the plane of the skin. A variety of wave patterns is known to occur in skin (Békésy, 1955; Keidel & Schmitt, 1955).

Mechanical events can explain many of the observations described, entirely or in part. It must, however, be remembered that the properties of the receptors themselves are affecting the results. The most important of these are the summation (Alvarez-Buylla & Ramirez de Arellano, 1953; Gray & Sato, 1953) and depression (Gray & Sato, 1953; Diamond, Gray & Inman, 1958; Loewenstein & Altamirano-Orrego, 1958) of the receptor potential. Furthermore, there are the properties of the conducting part of the receptor unit, refractoriness and the interaction of impulses at junctions (Katz, 1950; Hughes & Wiersma, 1960). There are two observations which require comment here. The summation between the excitatory effects of the on-and-off movements of a single pulse (Fig. 3) could be mechanical, but the effect is longer than the likely half-period of the mechanical change postulated above (*ca.* 0·6 msec), and it seems probable that this effect is due to summation of the receptor potentials (see Gray & Sato, 1953, Fig. 14). The later parts of the interaction curves can also be explained by an extension of the mechanical hypothesis, but it is not improbable that depression and refractoriness play their part.

A single unit can signal that a certain amplitude of displacement has been exceeded, provided the velocity is high enough. Alternatively, if the amplitude of a series of displacements is considerably greater than the critical amplitude, then the unit can distinguish those with a velocity above

threshold from those with lower velocities. A single pulse applied to an area of less than half a square millimetre can excite or influence receptor units across the pad or even on another pad; the amplitude and velocity required for excitation and the latency increase with distance. These facts must be taken into account in determining the system of coding. Stimulus strength (either amplitude or velocity of displacement, or both) cannot be signalled by frequency coding under these conditions; however, with bigger stimuli the number of units activated increases, because of the greater distance that a large stimulus can spread and remain above threshold; work on this system still to be published (C. J. Armett, J. A. B. Gray, R. W. Hunsperger and S. Lal) indicates that stimulus strength can be coded by the number of primary receptor units activated. The position of a stimulus could only rarely be signalled by the firing of a single unit, but it could be signalled by the mean position of the population. Timing factors may also play a part.

The interactions which follow two pulses may also be of functional significance. Any pacing or exploratory movement of the pad is likely to encounter a complex pattern of stimuli, and these results show that complex interactions which are critically dependent on timing can take place. A dramatic example is the inability of two pulses, each above threshold when alone, to excite when applied at a suitable interval.

These experiments are also a necessary basis to any work in which double mechanical pulse techniques are used to investigate the way in which signals converge upon and are analysed by second or higher-order neurones. False conclusions about the role of a group of junctions may be drawn if these interactions are not taken fully into account.

## SUMMARY

1. The activity produced in single receptor units, on mechanical stimulation of the cat's pad, has been recorded.

2. Both the displacement at the beginning and the restoration movement at the end of a mechanical pulse can excite or contribute to the excitation of the receptors. Excitation is determined by an amplitude required for threshold (range 1·5–16·5 $\mu$) and a critical slope (mean, 1·6 × amplitude required for threshold per millisecond).

3. Receptive fields have dimensions which are negatively correlated to the minimum threshold. Both the amplitude required for threshold ($\mu$) and the velocity required for threshold ($\mu$/msec) increase with distance from the centre of the field; critical slope (amplitude required for threshold/msec) is constant. The initiation of impulses is delayed at the periphery of the field.

4. Major changes in the excitability of a receptor to a test pulse on the main pad often result when a subthreshold conditioning pulse is applied at a second point on the main pad, or even occasionally on a small pad. These changes in excitability can be greater than a factor of two and can be of either sign; the precise effect is critically dependent on timing, particularly when the pulses are applied at intervals shorter than 1 msec.

5. Comparable changes in excitability can occur even when the conditioning pulse is applied to an area of pad which has been cut out and stuck back.

6. This and other evidence which is presented indicate that the properties of the receptive fields and the interactions may be explained by a mechanical wave that travels across the pad with a velocity of the order of 12 m/sec. This hypothesis is supported by certain mechanical observations which were made.

7. The results need to be taken into account in considering the coding of information by this population of receptor units. Furthermore, the results indicate that facilitatory and inhibitory functions should not be attributed to central mechanisms until there is reason to exclude the possibility of more peripheral interactions.

We wish to thank Professor J. A. B. Gray for his help during the final stages of the experimental work and in the preparation of the paper. C.A. was in receipt of a research training grant from the Medical Research Council and R.W.H. of a grant from the Swiss Academy of Science.

## REFERENCES

ALVAREZ-BUYLLA, R. & RAMEREZ DE ARELLANO, J. (1953). Local responses in Pacinian corpuscles. *Amer. J. Physiol.* **172**, 237–244.

VON BÉKÉSY, G. (1955). Human skin perception of travelling waves similar to those on the cochlea. *J. acoust. Soc. Amer.* **27**, 830–841.

DIAMOND, J., GRAY, J. A. B. & INMAN, D. R. (1958). The depression of the receptor potential in Pacinian corpuscles. *J. Physiol.* **141**, 117–131.

FERNANDEZ DE MOLINA, A. & GRAY, J. A. B. (1957). Activity in the dorsal spinal grey matter after stimulation of cutaneous nerves. *J. Physiol.* **137**, 126–140.

GRAY, J. A. B. (1959). Initiation of impulses at receptors. Chap. IV in *Handbook of Physiology*, Sect. 1, Neurophysiology. Ed. FIELD, J. Washington, D.C.: The American Physiological Society.

GRAY, J. A. B. & MALCOM, J. L. (1950). The initiation of nerve impulses by mesenteric Pacinian corpuscles. *Proc. Roy. Soc.* B, **137**, 96–114.

GRAY, J. A. B. & MALCOLM, J. L. (1951). The excitation of touch receptors in frog's skin. *J. Physiol.* **115**, 1–15.

GRAY, J. A. B. & MATTHEWS, P. B. C. (1951). A comparison of the adaptation of the Pacinian corpuscle with the accommodation of its own axon. *J. Physiol.* **114**, 454–464.

GRAY, J. A. B. & SATO, M. (1953). Properties of the receptor potential in Pacinian corpuscles. *J. Physiol.* **129**, 594–607.

HUBBARD, S. J. (1958). A study of rapid mechanical events in a mechanoreceptor. *J. Physiol.* **141**, 198–218.

HUGHES, G. M. & WIERSMA, C. A. G. (1960). Neuronal pathways and synaptic connexions in the abdominal cord of the crayfish. *J. exp. Biol.* **37**, 291–307.

KATZ, B. (1950). Action potentials from a sensory nerve ending. *J. Physiol.* **111**, 248–260.

KEIDEL, W. D. & SCHMITT, H. G. (1955). Hautwellenlängen und dynamischer Scherelastizitätskoeffizient der menschlichen Körperoberfläche bei Vibrationen mit 50 Hz. *Pflüg. Arch. ges. Physiol.* **260**, 274–291.

LOEWENSTEIN, W. R. (1956). Excitation and changes in adaptation by stretch of mechanoreceptors. *J. Physiol.* **133**, 588–602.

LOEWENSTEIN, W. R. & ALTAMIRANO-ORREGO, R. (1958). The refractory state of the generator and propagated potentials in a Pacinian corpuscle. *J. gen. Physiol.* **41**, 805–824.

MARUHASHI, J., MIZUGUCHI, K. & TASAKI, I. (1952). Action currents in single afferent nerve fibres elicited by stimulation of the skin of the toad and of the cat. *J. Physiol.* **117**, 129–151.

*J. Physiol.* (1961), **158**, *pp.* 39–49
*With 5 text-figures*
*Printed in Great Britain*



# THE CONTROL OF THE HEPATIC ARTERIAL CIRCULATION

By H. B. TORRANCE*

*From the Department of Surgical Science, University of Edinburgh*

(*Received* 13 *January* 1961)

The classical studies of Burton-Opitz (1910) laid the foundation of modern knowledge concerning the physiological behaviour of the arterial circulation of the liver, and despite sporadic attempts to gain more intimate information about it a significant advance did not occur until recently, when Grayson and his co-workers pursued a detailed investigation into the hepatic circulation in the rat (Grayson, 1952; Ginsburg & Grayson, 1954). The present investigations were undertaken to obtain more accurate data on the relationship between perfusion pressure and hepatic arterial blood flow in the dog.

## METHODS

Twenty-five healthy dogs varying in body weight between 9 and 25 kg were used in these experiments. Anaesthesia was induced by intravenous injection of sodium pentobarbital (30 mg/kg) and a free airway was maintained by an endotracheal tube.

*Operative procedures.* The abdomen was opened through an upper midline incision. Complete haemostasis was achieved either by diathermy coagulation or by ligature. The hepatic artery was exposed either in the free edge of the lesser omentum at a point distal to the origin of the gastro-duodenal artery, or at its origin from the coeliac artery, after ligation and division of the gastro-colic omentum. The periarterial nerve plexus was then carefully reflected, an occasional nerve fibre being sacrificed in order to expose approximately 2 cm of the artery. Collateral arterial vessels to the liver were then sought and the gastro-duodenal artery, the left gastric artery and the small arteries to the lesser curvature of the stomach were ligated. In a few experiments the common bile duct was ligated in order to occlude the fine arterial plexus which surrounds the duct and which may anastomose at a higher level with other branches of the hepatic artery.

Before cannulation of the various vessels, heparin ('Liquemin', Roche Products, Ltd, 1·9 mg/kg body wt.) was injected intravenously. The proximal portion of the exposed hepatic artery was ligated and after division of the vessel a wide-bore glass cannula was inserted into the distal cut end, a procedure facilitated by the application to the vessel wall of a small quantity of pethidine (50 mg/ml.) which eliminated spasm produced by manipulation of the vessel.

In a few experiments both ends of the divided artery were cannulated, blood flow being re-established through a flow-meter, but in the majority the inflow connexion of the flow-meter was connected to a wide-bore polyethylene cannula inserted into the aorta through a femoral artery. The complete circuit is shown in Fig. 1.

* Present address: Manchester Royal Infirmary, Oxford Road, Manchester 13.

*Blood-flow estimation.* The rate of hepatic arterial blood flow was measured by the density flowmeter described by Dawes, Mott & Vane (1953). In place of a mechanical time base and electromagnetic counter used by these authors, the time taken for 4 ml. of blood to flow through the metering chamber was measured in hundredths of seconds by a scaling unit with an A.C. mains time base (50 c/s). The apparatus was found to be capable of measuring blood flows up to 175 ml./min, with an accuracy of $\pm 2\%$. The pressure drop across the cannulae and the meter in a series of experiments *in vitro* using blood was found to be 10–15 mm Hg at 100 ml./min.

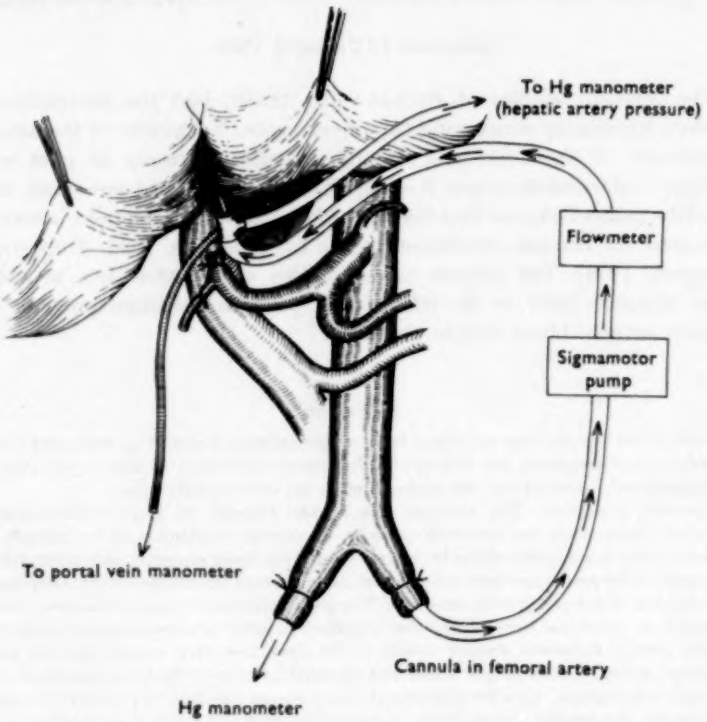



Fig. 1. Details of the methods employed to measure hepatic arterial blood flow and pressure.

*Pressure measurements.* Arterial blood pressures were recorded on a kymograph by mercury manometers. Systemic arterial pressure was recorded from a cannula tied into a femoral artery, and hepatic arterial pressure from a narrow side arm in the arterial cannula. The anterior surface of the abdominal aorta was taken as the zero reference point for arterial pressure measurements. In certain experiments portal venous pressure was measured from a polyethylene cannula inserted into the pancreatico-duodenal vein and recorded either by a saline manometer or by a new type of recording manometer (Simpson & Torrance, 1959). The ventral aspect of the vena cava was taken as the zero-pressure reference point.

*Determination of pressure–flow curves.* In the majority of experiments a Sigma motor pump, model TS6 with a Zero-Max control, was incorporated in the circuit proximal to the

flowmeter so that the rate of blood flow could be controlled accurately within wide limits. In a few experiments hepatic arterial perfusion pressure was varied by the application of a screw clip to the input connexion of the flowmeter.

In all experiments the absence of collateral vessels was confirmed by temporary occlusion of the circuit.

Following each alteration in perfusion pressure, a few moments were allowed for conditions to become stabilized. The mean flow rate was then determined from a series of at least six blood-flow measurements.

## RESULTS

### *Relationship between perfusion pressure and blood flow in the hepatic arterial system*

The relationships between perfusion pressure and rate of blood flow were studied in twenty-five experiments in which absence of significant collateral channels was confirmed. Two experiments were abandoned because large collateral vessels could not be controlled.

For purposes of analysis the initial pressure–flow curves from each individual experiment were selected. They showed that a non-linear relationship existed between perfusion pressure and blood flow in the hepatic arterial system. In general the curves showed a predominant convexity towards the flow axis, but in two instances curves convex to the pressure axis were found; in both experiments hepatic arterial resistance was unusually high—a finding which could not be accounted for by a mechanical defect in the perfusion circuit. In six experiments curves of definitely sigmoid shape were found, the apex of the convexity towards the pressure axis being situated around 40 mm Hg and the apex of the convexity towards the flow axis in the region of 70–80 mm Hg. In no instance did inflexions in the pressure–flow curves bear any relation to the systemic arterial pressure at the time of determination and this was taken as further evidence that collateral vessels had been satisfactorily occluded. In seventeen experiments the curves were purely convex towards the flow axis.

The effects of rapid increase and decrease in perfusion pressure were studied but the changes in blood flow were similar to those following gradual alteration in perfusion pressure, and hysteresis effects could not be demonstrated by the technique employed.

So that a standardized pressure–flow curve of the hepatic arterial system could be obtained, the results from each experiment were expressed as arterial flow per 100 g liver at the various levels of arterial pressure. Statistical justification for the expression of arterial flow in terms of unit of liver weight is considered later. The standardized curve, with the standard deviation at each pressure level, is shown in Fig. 2. The curve is obviously convex towards the flow axis, the apex of the convexity being

situated around 90 mm Hg. Around 40 mm Hg the curve deviates perceptibly towards the pressure axis, resulting in a sigmoid curve. The mean arterial perfusion pressure at which hepatic arterial flow fell to zero, i.e. the 'critical closing pressure' was found to be 18 mm Hg, S.D. ±5·8. Significant differences between critical closing pressure and critical opening pressure were not demonstrated.

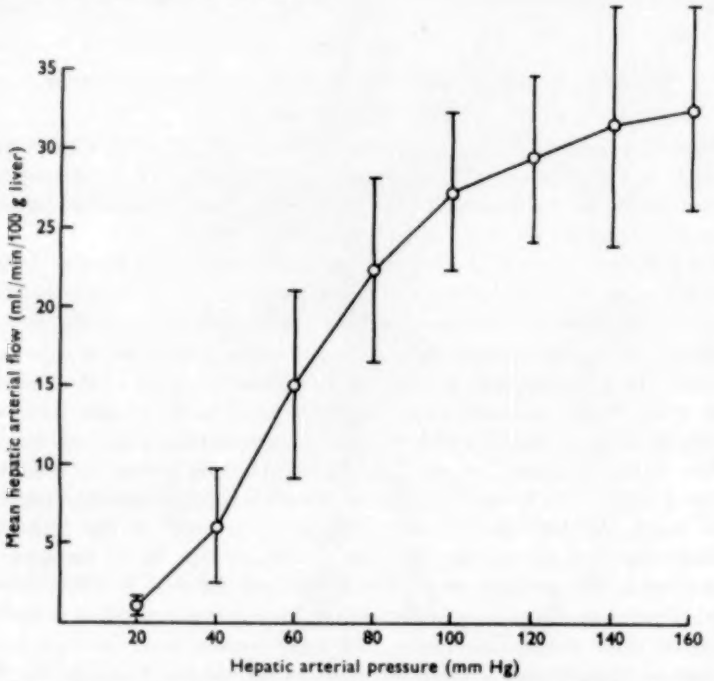



Fig. 2. The standardized pressure–flow diagram of the hepatic arterial circulation. The mean values and standard deviations were derived from twenty-five individual experiments. The form of this curve correlates closely with the results from individual experiments.

### *The effects of varying periods of hepatic arterial occlusion on hepatic arterial resistance*

Lest the non-linear relationships between hepatic arterial pressure and blood flow were due in part to the effects of the subnormal inflow on the liver, the results of different periods of arterial occlusion were studied. The inflow connexion to the flowmeter was occluded for intervals varying from 1 to 10 min; the hepatic arterial resistance before and after this procedure was then calculated, but no change was detected. The inhalation of a

mixture of 5% $CO_2$+95% $O_2$ has been shown in other experiments (Torrance, 1959) to lead to a profound fall in mesenteric vascular resistance, but this did not affect hepatic arterial resistance. It seems unlikely, therefore, that the increase in vascular resistance at the lower pressure and flow levels is due to the local effects of hypoxia on the hepatic circulation.

### *The effect of ganglion blockade and atropinization on the hepatic arterial pressure–flow relationships*

In four experiments ganglion blockade was produced by intravenous injection of hexamethonium bromide (25 mg). Comparison of the pressure–flow curves obtained 20 min after injection with control curves showed a deviation towards the flow axis in three out of four experiments, but the general shape of the curves was not affected (Fig. 3).

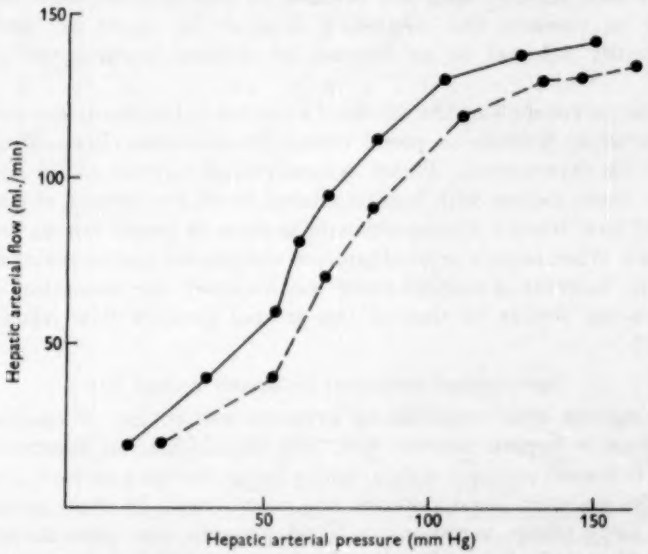



Fig. 3. Comparison of pressure–flow curves from an individual experiment before and after intravenous injection of hexamethonium bromide. Interrupted line indicates control curve.

The effects of an intravenous injection of atropine sulphate (3 mg) were also studied. In four separate experiments neither the form nor position of the pressure–flow curves was affected by the procedure.

It can be concluded, therefore, that the changes in vascular resistance induced by alterations in hepatic arterial pressure are not dependent on extrinsic autonomic nerve influences.

*Relationship between hepatic arterial flow and pressure and portal venous pressure*

In six experiments an attempt was made to determine the effect on portal venous pressure of variations in the hepatic arterial flow and pressure. Simultaneous measurement of portal venous flow by a rotameter and of hepatic artery flow was accomplished only in one experiment, owing to the technical difficulties involved.

In every instance portal venous pressure fell when the hepatic arterial circulation was occluded, although the fall was by no means uniform. The mean decrease in pressure was 1·5 cm of saline, with a range of 0·9–2·5 cm of saline, S.D. ±0·47.

When hepatic arterial flow was varied, portal venous pressure increased or decreased directly with the changes in hepatic-artery flow but the change in pressure was frequently delayed for 30–60 sec, and was occasionally followed by an increase or decrease towards the control value.

Stable curves showing the effects of variation in hepatic artery flow and hepatic artery pressure on portal venous pressure were obtained in three out of six experiments. Portal venous pressure varied in an approximately linear fashion with hepatic arterial blood flow, except at maximal rates of flow, when a disproportionate increase in portal venous pressure occurred. When hepatic arterial pressure was plotted against portal venous pressure, however, a sigmoid curve was obtained, the orientation of the curve being similar to that of the arterial pressure–flow relationship (Fig. 4).

*Spontaneous variations in hepatic arterial flow*

Throughout these experiments evidence was sought of spontaneous variations in hepatic arterial flow, but hepatic arterial resistance was found to remain constant within narrow limits over long periods, provided that the systemic blood pressure was maintained. In those animals in which large phasic variations in blood pressure were present, hepatic-artery flow showed corresponding fluctuations. When such variations in hepatic arterial flow were compared with a control pressure–flow curve, it was obvious that they could be accounted for entirely by the passive effect of the rhythmic alterations in arterial pressure.

*Correlation of hepatic arterial flow with liver weight*

At the conclusion of the experiments the liver was removed and weighed, no attempt being made to prevent blood loss from the major blood vessels. The mean liver weight in this series of experiments was found to be 2·26 %

of the body weight, a figure in close agreement with that obtained by Grindlay & Bollmann (1952).

In order to investigate the relationships between hepatic-artery flow (HAF) and liver weight (LW), the arterial flow within a closely defined pressure range 100–110 mm Hg was selected for the purposes of comparison. Statistical examination showed a highly significant correlation between hepatic arterial flow and liver weight ($t^2 = 12{\cdot}21$, $P < 0{\cdot}005$).

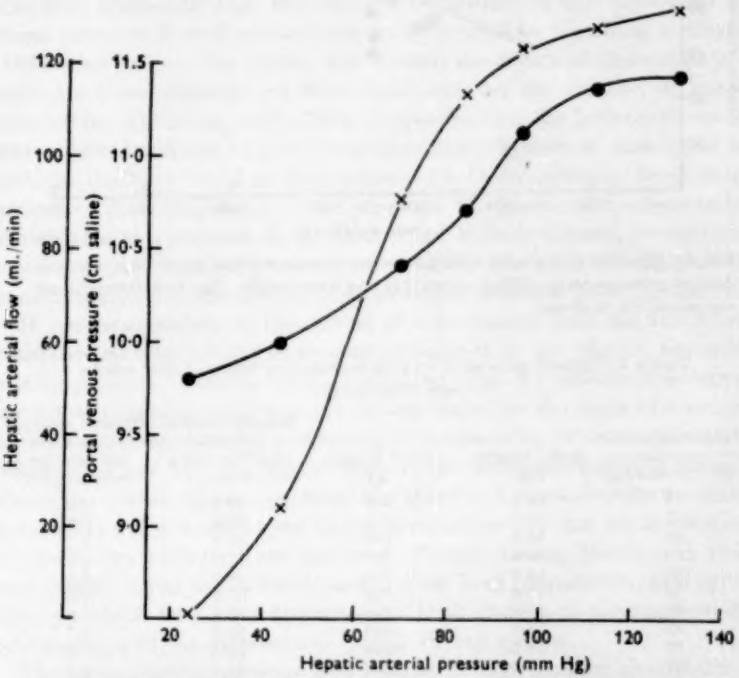



Fig. 4. In three experiments the relationships between portal venous pressure (●) and hepatic arterial pressure was sigmoid, probably indicating a relationship with the sigmoid hepatic arterial pressure–flow curve (×). The results of one of these experiments are demonstrated.

A linear regression line was calculated, the best fitting line being expressed by the formula HAF = 0·166 LW + 38·69 (Fig. 5). The significant correlation between arterial flow and liver weight provides reasonable justification for expressing hepatic arterial flow in terms of liver weight as used in the calculation of the standardized pressure–flow curve.

The relationships between hepatic arterial flow, liver weight and body weight are shown in Table 1. Mean hepatic arterial flow, expressed in

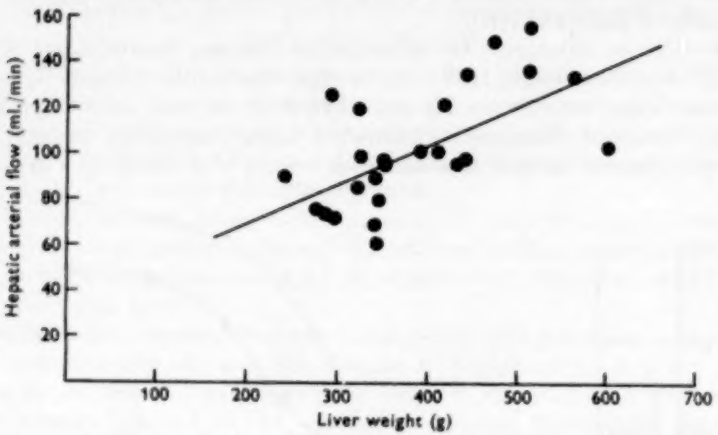



Fig. 5. Illustrating the relationship between hepatic arterial blood flow within a defined pressure range (100–110 mm Hg) and liver weight. The best fitting linear regression line is shown.

TABLE 1. Hepatic arterial flow rates expressed in terms of liver weight and body weight

| Hepatic arterial flow (ml./min) at 100–110 mm Hg | Body weight (kg) | Liver weight (g) | Hepatic arterial flow (ml./min/100 g liver) | Hepatic arterial flow (ml./min/kg body weight) |
|---|---|---|---|---|
| 75 | 14·5 | 285 | 26·3 | 5·2 |
| 122 | 14·0 | 414 | 29·4 | 8·7 |
| 66 | 16·0 | 400 | 16·5 | 4·1 |
| 120 | 14·0 | 320 | 37·5 | 8·6 |
| 101 | 14·0 | 390 | 26·0 | 7·1 |
| 75 | 10·0 | 275 | 27·2 | 7·5 |
| 90 | 11·6 | 240 | 37·4 | 7·6 |
| 80 | 14·0 | 345 | 23·2 | 5·7 |
| 98 | 14·0 | 350 | 28·0 | 7·0 |
| 126 | 14·0 | 290 | 43·4 | 9·0 |
| 72 | 15·6 | 293 | 24·5 | 5·8 |
| 61 | 10·0 | 345 | 17·7 | 6·1 |
| 99 | 25·0 | 440 | 22·5 | 3·6 |
| 102 | 17·5 | 410 | 23·7 | 6·0 |
| 86 | 16·5 | 320 | 27·0 | 5·2 |
| 95 | 16·0 | 430 | 22·3 | 5·8 |
| 90 | 11·0 | 340 | 26·8 | 8·2 |
| 134 | 16·0 | 560 | 24·0 | 8·4 |
| 138 | 17·5 | 507 | 27·6 | 8·0 |
| 105 | 26·0 | 600 | 17·5 | 4·0 |
| 149 | 16·0 | 470 | 32·0 | 9·3 |
| 100 | 13·2 | 325 | 30·8 | 7·6 |
| 135 | 14·0 | 440 | 30·7 | 9·6 |
| 96 | 13·4 | 350 | 27·4 | 7·2 |
| 156 | 14·6 | 510 | 31·0 | 10·1 |
| | | | Mean = 27·2 | Mean = 6·9 |
| | | | S.D. ± 5·6 | S.D. ± 1·9 |

terms of units of liver weight, was found to be 27·2 ml./min/100 g liver weight, S.D. = ±5·6. Mean hepatic arterial flow expressed in terms of body weight was 6·9 ml./min/kg body weight, S.D. = ±1·9.

## DISCUSSION

It has been recognized for some time that the relationship between perfusion pressure and blood flow in many vascular beds is non-linear. The first indication that the splanchnic circulation also possessed non-linear pressure–flow characteristics was provided by Ginsburg & Grayson (1954), and by Johnson (1954), who studied the effects of alterations in the systemic blood pressure on liver blood flow by the method of internal calorimetry (Grayson, 1952). They concluded that the hepatic circulation was endowed with an intrinsic auto-regulatory system so that blood flow through the liver could be maintained at a fairly constant level despite wide variations in systemic blood pressure. The present experiments have shown that the pressure–flow curve of the hepatic arterial circulation in the dog is predominantly convex towards the flow axis, a finding which is in accordance with the results of Ginsburg & Grayson (1954) in the rat.

It has been shown in this series of experiments that hepatic arterial resistance reaches infinity at a mean pressure of 18 mm Hg, i.e. the critical closing pressure (Burton, 1951). A sigmoid type of pressure–flow curve in the hepatic arterial system could be explained on the basis of a series of parallel vascular channels possessing critical closing pressures distributed between 18 and 100 mm Hg, so that as the perfusion pressure increased above the critical opening pressure the opening of extra circuits would lead to a steady fall in vascular resistance, until above 100 mm Hg the majority of circulatory pathways are perfused. Brauer, Leong, McElroy & Holloway (1956), using the isolated perfused rat liver preparation, also applied this hypothesis to explain the results of their studies on the pressure–flow relationships in the intrahepatic portal venous system.

The curve relating pressure and volume in large isolated elastic arteries such as the aorta has been shown to be sigmoid in shape (Roy, 1880; Hallock & Benson, 1937). Furthermore, the response of the resistance vessels to varying intraluminal pressure may also be determined by the response of the intramural smooth-muscle fibres. It has been suggested that when the pressure is increased the tendency to passive distension of the vessel is opposed by a myogenic constrictor reaction in the vessel wall (Bayliss, 1902; Folkow, 1949; Patterson & Shepherd, 1954; Coles & Greenfield, 1956). The evidence in favour of this concept has been accumulated entirely from studies of the circulation of the limb, and may not apply to the vessels of the visceral circulation. The anomalous viscosity properties of blood (Bayliss, 1952) are also likely to influence the pressure–

flow pattern to a considerable degree. Unfortunately it is not possible to determine from the present data which of these various factors is responsible for the non-linear pressure–flow relationships in the hepatic arterial circulation.

Using a mechanical stromuhr, Burton-Opitz (1910) estimated hepatic arterial flow to be 26 ml./min/100 g liver and 7·4 ml./min/kg body weight. McLeod & Pearce (1914) estimated hepatic arterial flow by the effects of occlusion of the hepatic artery on hepatic venous outflow and derived a mean value of 31 ml./min/100 g liver. Grodins, Osborne, Ivy & Goldman (1941) obtained a similar result (26 ml./min/100 g liver) using a thermostromuhr. The mean hepatic arterial flow in the present experiments was 27·2 ml./min/100 g liver, which agrees closely with the results of these earlier workers. On the other hand, the results obtained by Grab, Janssen & Rein (1929) and Grindlay, Herrick & Mann (1941) using thermostromuhrs, and Blalock & Mason (1937) using a collection method, indicated a much smaller contribution of hepatic arterial flow to liver blood flow. It seems likely, however, that the methods employed to estimate the rate of arterial flow were inadequate and liable to error from extraneous uncontrolled factors.

## SUMMARY

1. A method has been developed to study the dynamic behaviour of the hepatic arterial circulation in the dog.

2. The pressure–flow relationships in the hepatic arterial system were found to be non-linear and either predominantly convex to the flow axis or sigmoid.

3. Ganglion blockade and atropinization did not affect the general shapes of the pressure–flow curves.

4. The effects of variations in hepatic arterial blood flow and pressure on portal venous pressure were studied.

This work was carried out during the tenure of a Scottish Hospitals Endowment Research Trust Fellowship. I should like to express my gratitude to Dr B. Woolf and to Dr Donald Michie for the statistical calculations, to Mr G. Brooks and Mr Eric Leask for expert technical assistance and to Dr David Simpson of the Medical Physics Unit, University of Edinburgh, for his constant helpful advice.

## REFERENCES

Bayliss, L. E. (1952). *Deformation and Flow in Biological Systems.* Ed. Frey-Wyssling, A. Amsterdam: North Holland Publishing Co.

Bayliss, W. M. (1902). On the local reactions of the arterial wall to changes of internal pressure. *J. Physiol.* **28**, 220–231.

Blalock, A. & Mason, M. F. (1937). Observations on the blood flow and gaseous metabolism of the liver of the unanaesthetised dog. *Amer. J. Physiol.* **117**, 328–334.

Brauer, R. W., Leong, G. F., McElroy, R. F. & Holloway, R. J. (1956). Haemodynamics of the vascular tree of the isolated rat liver preparation. *Amer. J. Physiol.* **186**, 537–542.

BURTON, A. C. (1951). On the physical equilibrium of small blood vessels. *Amer. J. Physiol.* **164**, 319–329.

BURTON-OPITZ, R. (1910). The vascularity of the liver. I. The flow of blood in the hepatic artery. *Quart. J. exp. Physiol.* **3**, 297–313.

COLES, D. R. & GREENFIELD, A. D. M. (1956). The reactions of the blood vessels of the hand during increases in transmural pressure. *J. Physiol.* **131**, 277–289.

DAWES, G. S., MOTT, J. C. & VANE, J. R. (1953). Density flowmeter. Direct method for measurement of rate of blood flow. *J. Physiol.* **121**, 72–79.

FOLKOW, B. (1949). Intravascular pressure as a factor regulating the tone of the small vessels. *Acta physiol. scand.* **17**, 289–310.

GINSBURG, M. & GRAYSON, J. (1954). Factors controlling liver blood flow in the rat. *J. Physiol.* **123**, 574–602.

GRAB, W., JANSSEN, S. & REIN, H. (1929). Über die Grösse der Leber durch Blutung. *Z. Biol.* **89**, 324.

GRAYSON, J. (1952). Internal calorimetry in the determination of the thermal conductivity and blood flow. *J. Physiol.* **118**, 54–72.

GRINDLAY, J. H. & BOLLMANN, J. L. (1952). Regeneration of the liver in the dog after partial hepatectomy. Role of the venous circulation. *Surg. Gynaec. Obstet.* **94**, 491–496.

GRINDLAY, J. H., HERRICK, J. F. & MANN, F. C. (1941). Measurement of the blood flow of the liver. *Amer. J. Physiol.* **132**, 489–496.

GRODINS, F. S., OSBORNE, S. L., IVY, A. C. & GOLDMAN, L. (1941). The effect of bile acids on hepatic blood flow. *Amer. J. Physiol.* **132**, 375–389.

HALLOCK, P. & BENSON, J. C. (1937). Studies on the elastic properties of human isolated aorta. *J. clin. Invest.* **16**, 595–602.

JOHNSON, D. H. (1954). The effect of haemorrhage and hypotension on the liver blood flow. *J. Physiol.* **126**, 413–433.

MCLEOD, J. J. R. & PEARCE, R. G. (1914). The outflow of blood from the liver as affected by variations in the condition of the portal vein and hepatic artery. *Amer. J. Physiol.* **35**, 87–105.

PATTERSON, G. C. & SHEPHERD, J. T. (1954). The blood flow in the human forearm following venous congestion. *J. Physiol.* **125**, 501–507.

ROY, C. S. (1880). The elastic properties of the arterial wall. *J. Physiol.* **3**, 125–159.

SIMPSON, D. C. & TORRANCE, H. B. (1959). A new type of recording manometer for clinical research. *J. R. Coll. Surg. Edin.* **4**, 253–256.

TORRANCE, H. B. (1959). *A study of certain anatomical and physiological factors concerned in the control of the hepatic circulation.* Ch.M. Thesis, University of Edinburgh.

*J. Physiol.* (1961), **158**, *pp.* 50–58
*With 3 text-figures*
*Printed in Great Britain*

# DIRECT DETERMINATION OF EXTRACELLULAR FLUID IN THE RAT HEART

By R. B. FISHER* and D. A. B. YOUNG†
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

When the isolated saline-perfused rat heart is removed from the cannula, transected, blotted and allowed to stand in a weighing bottle, clear fluid exudes from it after a time. As the amount of this fluid is far in excess of the vascular volume, it seemed probable that it was being squeezed out of the interstitial spaces when the muscle fibres went into rigor. This observation suggested that it might be possible to express the extracellular fluid from the heart by simple compression.

The work described in this paper shows that in defined conditions it is possible to express reproducibly from the rat heart a volume of fluid equal to the extracellular volume. The composition of this fluid indicates that it is not unaltered extracellular fluid. Nevertheless, the observations provide a justification for the belief that raffinose and sorbitol spaces can be identified with the volume of mobile extracellular water.

## METHODS

*Animals.* Albino rats of Wistar stock weighing between 200 and 300 g were used.

*Preparation of hearts.* Hearts were removed under ether anaesthesia. If they were to be perfused, the procedure of Bleehen & Fisher (1954) was used, with the modifications described by Fisher & Lindsay (1956) and Bronk & Fisher (1957). When raffinose or sorbitol was used it was added to the perfusate in a concentration of 1 g/100 ml. Hearts were divided into anterior and posterior halves by a single cut with a scalpel and lightly blotted on Whatman No. 54 paper before compression.

*Compression apparatus.* The apparatus is shown in Fig. 1. It consists of a Perspex cylinder with a screw cap at each end. A rubber toy balloon is drawn over a conical expansion at the end of a Perspex tube. The balloon is drawn through a hole in the bottom of the Perspex cylinder and the cone on the Perspex tube is held against a conical seating in the bottom of the cylinder by the lower screw cap. Two circular Perspex slabs lie in the cylinder, the upper of which is turned out on its upper surface and has two notches in its rim, so that air can be expelled from the cylinder past it and out through a central hole in the top screw cap. After being weighed the halves of the heart are placed between two 3 cm Whatman No. 54 paper circles backed on each side by three 3 cm No. 1 papers, and the whole sandwich is placed between the Perspex disks, and inserted into the cylinder. The top cap is screwed on and the balloon inflated to a known pressure. The balloon is connected through a three-

* Present address: Department of Biochemistry, University of Edinburgh.
† Present address: Rowett Research Institute, Bucksburn, Aberdeen.

way tap to the atmosphere on one side and to a compressed-air cylinder on the other. A side tube in the compressed-air line connects to a mercury manometer.

After compression for a measured time the heart residue is weighed. For analysis the heart and the papers are homogenized separately in a top-drive homogenizer. The extract of the paper can be separated by filtration through sintered glass. The heart extract is deproteinized with cadmium sulphate and NaOH (Fujita & Iwatake, 1931).

*Raffinose determination.* The method used was that of S. W. Cole (unpublished), which depends on the development of a Seliwanoff colour in prescribed conditions. The optical density was measured in a Unicam SP600 absorptiometer at 480 mμ.

*Sorbitol determination.* Sorbitol was estimated by the method described by Fisher & Lindsay (1956).

*Creatine determination.* The diacetyl method of Ennor & Stocken (1948) was used. Optical density was measured with the SP600 at 520 mμ.

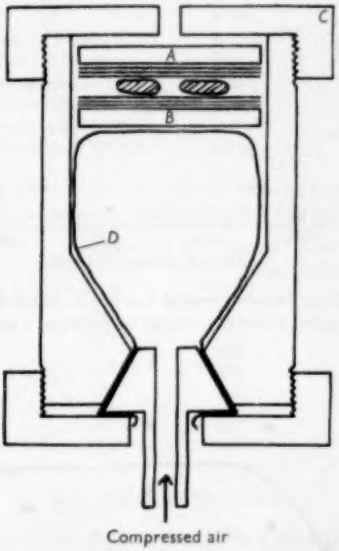



Fig. 1. Heart compression apparatus. The two halves of the heart are placed between the Perspex disks *A* and *B* resting on the balloon *D*, which is inflated after screwing on the cap *C*.

## RESULTS

*Conditions for the reproducible expression of fluid.* When pressure was applied for 3 min it was found that weight loss was not increased by raising the pressure above 500 mm Hg. The results are shown in Fig. 2. However, the weight loss in these experiments did not approach the figure of 250–280 mg/g wet weight which was to be expected if all the extracellular fluid had been expressed (Lowry, Hastings, Hull & Brown, 1942; Lemley & Meneely, 1952).

*J. Physiol.* (1961), **158**, *pp.* 50–58
*With* 3 *text-figures*
*Printed in Great Britain*

# DIRECT DETERMINATION OF EXTRACELLULAR FLUID IN THE RAT HEART

By R. B. FISHER* and D. A. B. YOUNG†
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

When the isolated saline-perfused rat heart is removed from the cannula, transected, blotted and allowed to stand in a weighing bottle, clear fluid exudes from it after a time. As the amount of this fluid is far in excess of the vascular volume, it seemed probable that it was being squeezed out of the interstitial spaces when the muscle fibres went into rigor. This observation suggested that it might be possible to express the extracellular fluid from the heart by simple compression.

The work described in this paper shows that in defined conditions it is possible to express reproducibly from the rat heart a volume of fluid equal to the extracellular volume. The composition of this fluid indicates that it is not unaltered extracellular fluid. Nevertheless, the observations provide a justification for the belief that raffinose and sorbitol spaces can be identified with the volume of mobile extracellular water.

## METHODS

*Animals.* Albino rats of Wistar stock weighing between 200 and 300 g were used.

*Preparation of hearts.* Hearts were removed under ether anaesthesia. If they were to be perfused, the procedure of Bleehen & Fisher (1954) was used, with the modifications described by Fisher & Lindsay (1956) and Bronk & Fisher (1957). When raffinose or sorbitol was used it was added to the perfusate in a concentration of 1 g/100 ml. Hearts were divided into anterior and posterior halves by a single cut with a scalpel and lightly blotted on Whatman No. 54 paper before compression.

*Compression apparatus.* The apparatus is shown in Fig. 1. It consists of a Perspex cylinder with a screw cap at each end. A rubber toy balloon is drawn over a conical expansion at the end of a Perspex tube. The balloon is drawn through a hole in the bottom of the Perspex cylinder and the cone on the Perspex tube is held against a conical seating in the bottom of the cylinder by the lower screw cap. Two circular Perspex slabs lie in the cylinder, the upper of which is turned out on its upper surface and has two notches in its rim, so that air can be expelled from the cylinder past it and out through a central hole in the top screw cap. After being weighed the halves of the heart are placed between two 3 cm Whatman No. 54 paper circles backed on each side by three 3 cm No. 1 papers, and the whole sandwich is placed between the Perspex disks, and inserted into the cylinder. The top cap is screwed on and the balloon inflated to a known pressure. The balloon is connected through a three-

* Present address: Department of Biochemistry, University of Edinburgh.
† Present address: Rowett Research Institute, Bucksburn, Aberdeen.

way tap to the atmosphere on one side and to a compressed-air cylinder on the other. A side tube in the compressed-air line connects to a mercury manometer.

After compression for a measured time the heart residue is weighed. For analysis the heart and the papers are homogenized separately in a top-drive homogenizer. The extract of the paper can be separated by filtration through sintered glass. The heart extract is deproteinized with cadmium sulphate and NaOH (Fujita & Iwatake, 1931).

*Raffinose determination.* The method used was that of S. W. Cole (unpublished), which depends on the development of a Seliwanoff colour in prescribed conditions. The optical density was measured in a Unicam SP 600 absorptiometer at 480 mμ.

*Sorbitol determination.* Sorbitol was estimated by the method described by Fisher & Lindsay (1956).

*Creatine determination.* The diacetyl method of Ennor & Stocken (1948) was used. Optical density was measured with the SP 600 at 520 mμ.

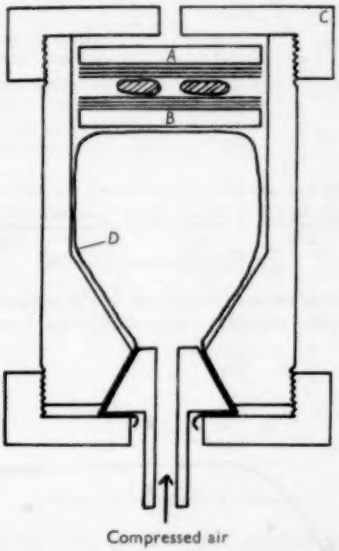



Fig. 1. Heart compression apparatus. The two halves of the heart are placed between the Perspex disks *A* and *B* resting on the balloon *D*, which is inflated after screwing on the cap *C*.

## RESULTS

*Conditions for the reproducible expression of fluid.* When pressure was applied for 3 min it was found that weight loss was not increased by raising the pressure above 500 mm Hg. The results are shown in Fig. 2. However, the weight loss in these experiments did not approach the figure of 250–280 mg/g wet weight which was to be expected if all the extracellular fluid had been expressed (Lowry, Hastings, Hull & Brown, 1942; Lemley & Meneely, 1952).

A second set of trials was made using different times of compression at 500 mm Hg. The results are shown in Fig. 3. These data show that weight loss ceases after approximately 15 min and that the magnitude of the maximal loss corresponds to that of the extracellular fluid.

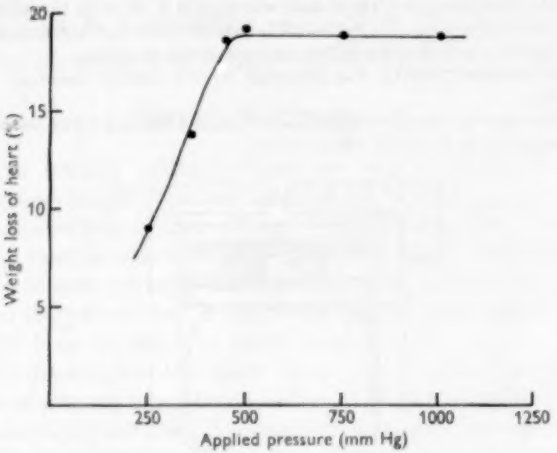



Fig. 2. The relation between weight loss of the compressed heart and applied pressure. Compression time, 3 min.

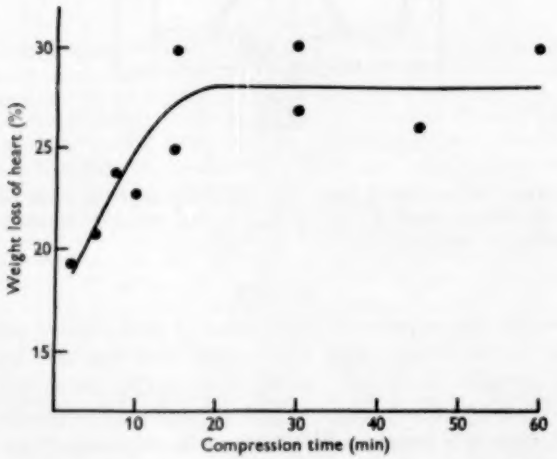



Fig. 3. The relation between weight loss of the compressed heart and compression time. Pressure = 500 mm Hg.

In a larger series of trials one half of each heart was compressed for 15 min at 500 mm Hg and the other half was compressed for 30 min. Two compression chambers were used and a trial on eight hearts was designed so that the effects of difference in time of compression, differences between anterior and posterior halves of the heart and differences between the first and second half of the heart to be put under compression could be analysed. The results are given in Table 1.

TABLE 1. The influence of compression time, order of compression and source of tissue on the weight loss of fresh rat heart on compression at 500 mm Hg pressure: eight experiments

| | Mean wt. loss (mg/g) | *P* |
|---|---|---|
| Anterior half of heart | 270·6 | 0·02 > 0·01 |
| Posterior half of heart | 301·6 | |
| 1st portion compressed | 296·3 | 0·1 > 0·05 |
| 2nd portion compressed | 276·0 | |
| Portion compressed 15 min | 275·5 | 0·1 > 0·05 |
| Portion compressed 30 min | 296·8 | |

TABLE 2. The relation between sorbitol space and weight loss on compression of hearts perfused for 30 min

| | Sorbitol space (μl./g) | Wt. loss on compression (mg/g) |
|---|---|---|
| | 348 | 324 |
| | 325 | 273 |
| | 350 | 359 |
| | 318 | 352 |
| | 309 | 280 |
| | 306 | 286 |
| | 338 | 285 |
| | 311 | 338 |
| Means | 325·6 | 312·6 |

Mean difference 13·5 ± 12·7

Analysis of variance showed that the difference between the two halves of the heart was on the borderline of significance, but that time of compression or the order in which the samples were compressed had no significant effect on the outcome. That is, time of compression beyond 15 min is not critical and delay between death and compression up to the 5 min which sometimes elapsed in these experiments is not of importance. The mean weight loss in these experiments was 286 ± 5 mg/g (S.E. of mean) for female rats weighing 200–300 g.

*Relation between weight loss and sorbitol space.* Perfusions lasting 30 min with Krebs's bicarbonate medium containing 1 g sorbitol/100 ml. were carried out on eight hearts. The results of determinations of sorbitol space and weight loss on compression are given in Table 2. It is clear that there is no systematic difference between the two sets of measurements.

*Relation between raffinose space and weight loss.* Series of hearts were perfused with Krebs's bicarbonate medium containing 1 g raffinose/100 ml. for 15, 30 and 60 min respectively. The results of raffinose space and weight loss measurements are given in Table 3. The correspondence here is better than in the sorbitol experiments: this is probably attributable to the difficulty of measuring small concentrations of sorbitol accurately.

TABLE 3. The relation between raffinose space and weight loss on compression of hearts perfused for various intervals of time

| Duration of perfusion (min) | Raffinose space (μl./g) | Wt. loss on compression (mg/g) |
|---|---|---|
| 15 | 342 | 338 |
| | 322 | 321 |
| | 330 | 349 |
| Means | 331 | 336 |
| Mean difference 4·3 ± 7·2 | | |
| 30 | 322 | 328 |
| | 358 | 337 |
| | 324 | 325 |
| | 358 | 354 |
| Means | 341 | 336 |
| Mean difference 4·5 ± 5·9 | | |
| 60 | 377 | 358 |
| | 383 | 372 |
| | 360 | 377 |
| | 389 | 380 |
| | 386 | 345 |
| | 397 | 374 |
| | 355 | 375 |
| | 381 | 353 |
| Means | 379 | 367 |
| Mean difference 11·8 ± 7·5 | | |

*The composition of the expressed fluid.* The correspondence between chemically determined extracellular space and weight loss gave ground for hope that separated samples of extracellular fluid could be obtained by this method. However, when estimations of raffinose in the expressed fluid were made, it was found that it contained significantly less than did the whole heart. In further studies the partition of raffinose and creatine between expressed fluid and heart residue was studied. Unpublished experiments had shown that in the conditions normally used for heart perfusion very little creatine is lost by the heart, the ratio of concentration in intracellular water to concentration in perfusate being of the order of a thousand to one. Thus raffinose and creatine constitute a pair of solutes one of which does not normally enter cells, whilst the other is normally retained in the cells. The results of these measurements are given in

Table 4. They show that during compression approximately 37 % of the raffinose has passed into the intracellular water whilst 37 % of the creatine has passed out.

TABLE 4. The partition of raffinose and creatine between perfusate, expressed fluid and residual heart water, for hearts perfused for 1 hr with 1 % raffinose

| | | Experiment | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| Creatine (μg/ml.) | Perfusate | 4·4 | 3·9 | 3·0 |
| | Expressed fluid | 1764·0 | 2053·0 | 1748·0 |
| | Residual water | 2192·0 | 2471·0 | 2557·0 |
| Raffinose (mg/ml.) | Perfusate | 9·81 | 16·15 | 10·29 |
| | Expressed fluid | 6·67 | 6·11 | 7·07 |
| | Residual water | 3·30 | 2·86 | 3·12 |
| Raffinose space (μl./g) | | 397 | 355 | 381 |
| Wt. loss on compression (mg/g) | | 374 | 375 | 353 |

37·1 % of the creatine has come out, and 36·5 % of the raffinose has gone into the cellular compartment.

TABLE 5. The influence of anoxia on the efflux of creatine from the cells of the perfused rat heart

Anoxia resulting from equilibration of perfusate with $N_2 + 5\%$ $CO_2$

| | Gas equilibration | Time (min) | Time of sampling | Creatine added during interval (μg) |
|---|---|---|---|---|
| Expt. 1 | $O_2 + 5\%$ $CO_2$ | 0–20 | 20 | 36 |
| | $N_2 + 5\%$ $CO_2$ | 20–25 | 31 | 20 |
| | $O_2 + 5\%$ $CO_2$ | 25–52 | 52 | 14 |
| Expt. 2 | $O_2 + 5\%$ $CO_2$ | 0–26 | 26 | 53 |
| | $N_2 + 5\%$ $CO_2$ | 26–36 | 41 | 3 |
| | $O_2 + 5\%$ $CO_2$ | 36–71 | 71 | 5 |
| Expt. 3 | $O_2 + 5\%$ $CO_2$ | 0–45 | 45 | 120 |
| | $N_2 + 5\%$ $CO_2$ | 45–60 | 65 | 20 |
| | $O_2 + 5\%$ $CO_2$ | 60–85 | 85 | 10 |
| Anoxia resulting from stasis | | | | |
| | $O_2 + 5\%$ $CO_2$ | 0–30 | 30 | 59 |
| | Stasis | 30–40 | 45 | 7 |
| | $O_2 + 5\%$ $CO_2$ | 40–60 | 60 | 7 |

*The effects of anoxia on creatine loss from the heart.* The experiments just described appeared to indicate a rapid post-mortem increase in cell permeability. Experiments were made to determine whether this was the result of the onset of anoxia. The effect of anoxia on the escape of creatine from the heart was determined, the anoxia being produced either by switching the equilibrating gas mixture from 95 % $O_2 + 5\%$ $CO_2$ to 95 % $N_2 + 5\%$ $CO_2$ or by clamping off the inflow of oxygenated perfusate to the heart. The results of these experiments are given in Table 5. Despite the irregularity of the results it is quite clear that there is no greater leak of creatine from the anoxic heart than from the aerobic heart.

Two 45 min perfusion experiments were made in which raffinose was perfused, with nitrogen as the equilibrating gas, to determine whether in these circumstances the raffinose space became larger than the weight loss on compression. In one experiment the raffinose space was 425 $\mu$l./g and weight loss 429, in the other raffinose space was 312 and weight loss 320. There is therefore no evidence that anoxia is a factor in changing cell permeability to creatine or raffinose.

*The water content of compressed hearts.* Confirmation of the view that weight loss on compression is due to expression of fluid from the extracellular space is given by measurement of the water content of the residue after compression. Table 6 gives such measurements, together with the weight loss on compression for fresh hearts, and hearts perfused for 30 min. The weight loss on compression is much greater for perfused hearts, but the water content of the residue is almost identical in the two series.

TABLE 6. The partition of water between expressed fluid and heart residue, for control hearts (unperfused) and hearts perfused for half an hour

| | Expressed fluid (g $H_2O$/g heart solid) | Residual water |
|---|---|---|
| Controls (8) | 1·274 ± 0·046 | 2·674 ± 0·03 |
| Perfused (8) | 2·145 ± 0·114 | 2·788 ± 0·03 |

## DISCUSSION

The results of this work show that the fraction of fresh heart weight lost on compression is reproducible, provided that the pressure and time of compression exceed certain minimal values. This in itself suggests that the weight loss corresponds to some definite structural fraction of the tissue. The fact that the weight loss of the fresh heart is equal in magnitude to the published estimates of the extracellular volume of the rat heart supports the identification of the fraction lost with the extracellular fluid.

The correspondence in magnitude between weight loss and previous estimates of the extracellular space cannot be expected to be perfect. The two series of observations on weight loss in fresh hearts which are given in Tables 1 and 6 do not agree with one another. This is a sex difference. The data of Table 1 give a mean of 286 ± 5 mg/g for eight female rats. The data of Table 6 give a mean of 257 ± 7 for eight male rats. Lemley & Meneely (1952) give 280 ± 21 $\mu$l./g for sodium space in 11 male rats of the Sprague–Dawley strain. Lowry *et al.* (1942) give 239 ± 4 $\mu$l./g as the chloride space of hearts of forty-seven rats of the Yale–Cornell strain; the sex is not stated.

It seems, therefore, that there are real differences in extracellular volume in the hearts of different strains and sexes of rat. Establishment of identity

of weight loss with extracellular space calls for chemical and compression measurements on the same heart.

The measurements reported here show that the loss on compression of the fresh heart corresponds to both the sorbitol and the raffinose spaces. It has also been shown that when the heart becomes oedematous during perfusion, as judged by the raffinose space, the compression loss increases correspondingly. It is clear, therefore, that the correspondence found in the fresh heart is not merely an arithmetical coincidence: the two kinds of measurement are measuring the same property of the heart.

It is to be expected that saline perfusion will render the heart oedematous. Since the heart continues to function quite well in our conditions for the whole period of perfusion it is unlikely that major disorganization of its cells will occur in this time. The partition of heart water between that lost on compression and that retained corresponds very plausibly on this basis with a partition between extracellular and intracellular. As can be seen from Table 6, the increase in the compression loss, when it is expressed with reference to heart solids, is large; in these experiments it is 68%. In contrast, the increase in residual water is small, amounting to 4%. Comparison with the data of other workers for intracellular water does not much reinforce the appeal to plausibility, however, for much the same reasons as those that have already been discussed in connexion with extracellular water. It is true that Lowry *et al.* (1942) derived an intracellular water estimate for their Yale–Cornell animals of 2·7 g/g solids, as compared with 2·67 for the data of Table 6, but the data of Lemley & Meneely (1952) yield a value of 2·0 g/g solids.

The importance of these experiments is that they bring to bear another kind of measure of the extracellular space. Even though the direct determination of extracellular space by compression may not recommend itself as a routine procedure, the fact that it gives results in a variety of conditions which agree with those obtained by two different chemical methods should increase confidence in the interpretation of the chemical methods.

This confidence should not be disturbed by the evidence presented for the passage of raffinose into the cells and for the passage of creatine out of them during the compression period. During compression all cells lying in the continuous extracellular phase can be taken to be subjected uniformly to increased pressure, since the rate of flow of fluid from the heart is low. As a consequence, intracellular and extracellular pressures should be equal. Thus, any increase of water influx of hydrostatic origin should be balanced by an equal increase in efflux. On the other hand, for solutes such as raffinose and creatine, which are asymmetrically distributed about the cell membrane, rise in hydrostatic pressure will accelerate net flux down the concentration gradient. However, since the absolute pressure

in the tissue rises by less than a factor of two during compression, there must be a permeability change to account for the rapid movement observed for these two solutes.

The important thing about the movements of raffinose and creatine is that they occur to just about the same extent in opposite directions, and thus confirm approximately the judgement made here that the flux of water, which is initially present in very similar concentrations on the two sides of the cell membrane, will be increased correspondingly in both directions as a result of the changes occurring during compression. That is, there should be negligible net water flux in either direction.

The outcome of this work is therefore that one can accept with greater confidence than hitherto the view that the sorbitol space and the raffinose space of the heart are good measures of the volume of extracellular fluid.

## SUMMARY

1. When the transected rat heart is compressed between filter papers at a pressure not less than 500 mm Hg and for a time not less than 15 min it loses weight to an extent independent of the pressure or time of compression.

2. The weight loss of perfused hearts corresponds in magnitude to the expected volume of extracellular fluid.

3. The weight loss of perfused hearts corresponds to the raffinose or sorbitol space of the heart.

4. The water content of the residue after compression is almost identical for fresh hearts and for hearts perfused for up to an hour.

5. When the heart has been perfused with raffinose, there is less of this in the fluid and much more creatine than there would be expected to be in extracellular fluid.

## REFERENCES

Bleehen, N. M. & Fisher, R. B. (1954). The action of insulin on the isolated rat heart. *J. Physiol.* **123**, 260–276.

Bronk, M. S. & Fisher, R. B. (1957). The interaction of growth hormone and insulin in the perfused rat heart. *J. Physiol.* **136**, 435–448.

Ennor, A. H. & Stocken, L. A. (1948). The estimation of creatine. *Biochem. J.* **42**, 557–563.

Fisher, R. B. & Lindsay, D. B. (1956). The action of insulin on the penetration of sugars into the perfused rat heart. *J. Physiol.* **131**, 526–541.

Fujita, A. & Iwatake, D. (1931). Bestimmung des echten Blutzuckers ohne Hefe. *Biochem. Z.* **242**, 43–60.

Lemley, J. M. & Meneely, G. R. (1952). Distribution of tissue fluid in hearts of rats subjected to anoxia. *Amer. J. Physiol.* **169**, 61–65.

Lowry, O. H., Hastings, A. B., Hull, T. Z. & Brown, A. N. (1942). Histochemical changes associated with ageing. II. Skeletal and cardiac muscle in the rat. *J. biol. Chem.* **143**, 271–280.

*J. Physiol.* (1961), **158**, *pp.* 59–72
*With 6 text-figures*
*Printed in Great Britain*



# CONTRACTILITY AND SUGAR PERMEABILITY IN THE PERFUSED RAT HEART

By P. ZACHARIAH*
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

The work to be described shows that the permeability of the rat heart to a non-metabolized sugar changes continuously during perfusion with Krebs-bicarbonate medium. Such a preparation is obviously unsuitable for the study of the kinetics of permeation of the cells by sugars. A preparation with more stable permeability properties is described which can be obtained by the use of a modified perfusion medium.

## METHODS

*Animals.* Male albino rats weighing between 220 and 280 g were used. They were kept for a few days before use in a room at a constant temperature of 23° C. They had free access to water and food.

*Operative procedure.* This was that described by Bronk & Fisher (1957), except that 4 mg heparin (British Drug Houses) dissolved in 1 ml. normal saline solution was injected intravenously 3 min before the excision of the heart.

*Perfusion technique.* Earlier workers in this laboratory (Bleehen & Fisher, 1954; Fisher & Lindsay, 1956; Bronk & Fisher, 1957) recirculated the perfusate continuously through cellulose extraction thimbles. In the course of studies on sugar efflux from perfused hearts D. A. B. Young in this laboratory found that significant portions of the heart were occasionally not perfused. This appeared to be due to inadequate filtration of the perfusate. A more effective filtration procedure, illustrated in Fig. 1, was therefore adopted. The perfusate leaving the heart was carried by a gas lift (5 % $CO_2$ in $O_2$) to a reservoir from which it passed under hydrostatic pressure to a sintered-glass filter with a pore size of 5–10 $\mu$ (No. 17, Gallenkamp). With a hydrostatic pressure of 75 cm water and a filter diameter of 7 cm, the rate of filtration was about 30 ml./min, i.e. about three times the maximum rate of perfusion. It was advisable to use an extraction thimble in the filter reservoir to delay blockage of the sintered-glass filter.

As is shown in Fig. 1, the effluent from the filter was returned to the perfusion reservoir by a second gas lift. The fluid level in the filter reservoir was slightly lower than in the perfusion reservoir; the excess of perfusate returned to the perfusion reservoir from the filter was transferred to the other reservoir by a siphon connecting the two. The heart perfusion pressure was 60 cm $H_2O$.

*Measurement of the rate and force of contraction of the heart.* In order to measure the force of contraction of hearts perfused by the closed-circuit technique, the heart chamber and accessories shown in Fig. 2 were devised. The valve in the outflow line from the heart

* Rockefeller Foundation Fellow. Present address: Department of Physiology, Christian Medical College, Vellore, S. India.

chamber was made of thin sheet rubber and this valve prevented the regurgitation of fluid into the heart chamber due to back pressure in the return tube leading to the filtration reservoir.

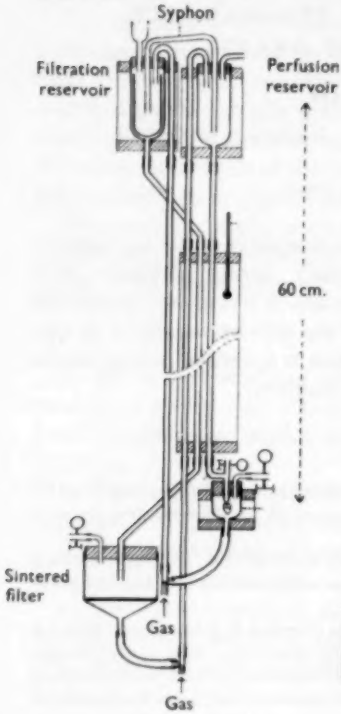



Fig. 1

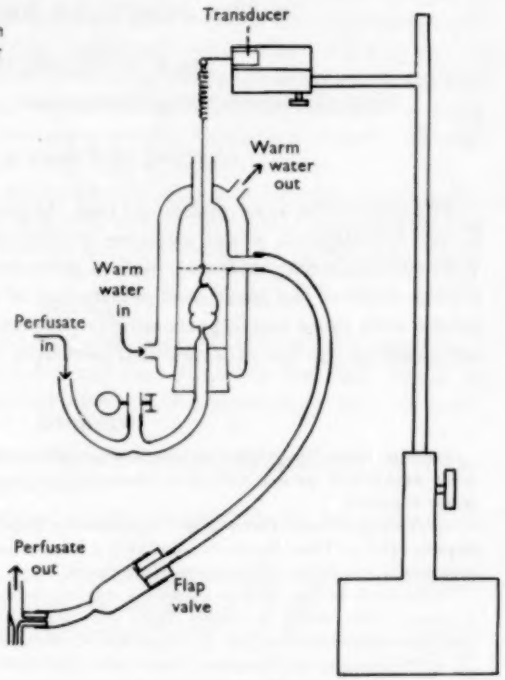



Fig. 2

Fig. 1. Closed-circuit perfusion unit with improved filtration apparatus.

Fig. 2. Accessories for recording the contractility of the heart perfused in the closed-circuit unit.

In the preliminary experiments the amplitude of contraction of the hearts was determined with a kymograph and isotonic lever. Later more precise measurements were made with a transducer (RCA 5734) and a cathode-ray oscillograph (A. C. Cossor Ltd., Model 1049, Mk. II). The heart was connected to the anode pin of the transducer through a fine steel spring, as shown in Fig. 2. The transducer was mounted on a stand with a vertical rack and pinion so that the tension of the spring could be adjusted easily. Normally the tension was adjusted to be equal to 1·5 g during diastole. The output of the transducer was used to trigger a thyratron coupled to an electromagnetic counter and the heart rate was determined on this at regular intervals.

*Perfusate.* The basic perfusate was Krebs-bicarbonate medium (Krebs & Henseleit, 1932), containing half of the concentration of calcium that was originally recommended.

*Determination of the sugar uptake by the heart.* On removal from the perfusion apparatus the heart was quickly blotted on hardened filter paper and weighed. It was then homo-

genized in 25 ml. of water. A portion of the homogenate was removed for dry weight determination and the proteins in the rest of the homogenate were precipitated with $CdSO_4$ and NaOH (Fujita & Iwatake, 1931). A sample of the perfusate was also removed at the end of perfusion and treated in the same manner as the homogenate. The filtrates were analysed for pentose and raffinose.

*Analytical techniques*

***Pentose estimation.*** A slightly modified version of the *p*-bromoaniline method of Roe & Rice (1948) was used. The temperature of the reaction was raised to 80° C and the intensity of colour was estimated between 90 and 130 min after removal of the tubes from the hot water-bath.

***Raffinose estimation.*** The method of S. W. Cole (unpublished), which is based on an adaptation of the Seliwanoff reaction, was used.

***Chloride estimation.*** The titrimetric method of Sendroy (1937) was used.

***Potassium estimation.*** A nitric-acid digest of the dried residue of the heart (*vide infra*) was analysed by flame photometry.

***Dry weight determination.*** A portion of the heart homogenate (equal to 2/5 of the total volume) was dried in an oven at 110° C for 12 hr and the weight of the residue determined. The dry weight of the whole heart was calculated from this. The coefficient of variation of dry weight determination by this method was 0·24 %.

*Calculations*

The extracellular space was taken to be equal to the raffinose space (Helmreich & Cori, 1957).

Intracellular water content = wet weight − (dry weight + extracellular space).

$$\text{Total sugar space} = \frac{\text{sugar content of the tissue}}{\text{sugar concentration in the perfusate}}.$$

Intracellular pentose space = total pentose space − extracellular space.

$$\text{Percentage penetration of cell water pentose} = \frac{\text{intracellular pentose space}}{\text{volume of intracellular water}}.$$

The mean of the results of a group of observations is presented as mean ± S.E. of mean (number of observations).

## RESULTS

***Rate of coronary flow.*** In a series of eleven hearts perfused by the method of Bleehen & Fisher (1954) it was found that the mean flow rate fell from 8·4 ml. to 5·5 ml./min/g wet wt. in the course of 45 min of perfusion. Comparable values for a set of seven hearts perfused at the same pressure head, but using the sintered-glass filter procedure, were 9·9 and 8·3 ml./min/g wet wt. In the latter series the flow rate at the end of 90 min was 6·2 ml./min/g wet wt. J. R. Williamson and R. B. Fisher (unpublished observations) have found that the oxygen consumption of the perfused rat heart at 37° C. is 37·5 ml./g dry wt./hr. This requires a perfusion rate of at least 5 ml./g wet wt./min of perfusate saturated with 95 % $O_2$. Thus the incorporation of the sintered-glass filter into the perfusion apparatus provides a better margin of safety with regard to the adequacy of the perfusion.

*Contractility*

It soon became clear that the force of contraction of hearts perfused by the new technique was falling off quite rapidly. After 45 min in all but a few hearts the beat appeared to be feeble. This was confirmed by measurement of the amplitude of contraction with a kymograph and an isotonic lever. A more detailed study of the pattern of the changes in the contractility of the perfused hearts was made with the aid of a transducer.

The force of contraction of the heart was determined at intervals of 5 min, the increase in the voltage of the output of the transducer being proportionate to the force applied to the anode pin by the contraction. As there was considerable individual variation in the absolute values determined in this way, the contractility of each heart was recorded as a percentage of the mean of the six determinations made during the first 30 min of perfusion; this mean value was taken to be the normal 'contractility' of that heart. The efficacy of any perfusate in providing a stable isolated-heart preparation could be estimated from the duration of 'normal' contractility in a group of three or more hearts perfused with the medium.

The contractility changes in four hearts perfused with Krebs-bicarbonate medium are shown in Fig. 3. The reproducibility of the changes is obvious. It is also seen (from this figure) that in a group of eight hearts perfused with this medium the duration of normal contractility was only 35 min. In these and subsequent experiments no correlation was observed between heart rate and the change in force of contraction. There was considerable variability in rate between hearts, but each heart maintained its rate fairly steadily throughout the period of observation. The majority of heart rates were in a range of 220–260 beats/min.

In the above experiments the concentration of calcium in the perfusate had been halved, as was the practice of previous workers in this laboratory and elsewhere (e.g. Boyle & Conway, 1941), in order to allow for the absence of the calcium-binding effect of the plasma proteins in the saline perfusate. However, magnesium is also bound by proteins to a similar extent (Greene & Power, 1931). Thus in these experiments the concentration of ionic magnesium was higher than in plasma, both in absolute terms and in comparison to the concentration of calcium. When the magnesium concentration was also reduced by half, the duration of normal contractility was extended to 55 min; small changes in the magnesium content of the perfusate around this value were of no effect. In all the subsequent experiments the concentration of calcium and magnesium in the perfusate was reduced to half that recommended by Krebs & Henseleit (1932). Sets of three to four control perfusions with this medium were

made at intervals throughout this investigation and have shown satisfactory reproducibility.

Attempts to delay the onset of deterioration of the heart by the provision of nutrients known to be utilized by the heart (glucose with insulin, succinate, lactate, pyruvate, acetoacetate) were unsuccessful. The duration of normal contractility could not be prolonged by reducing the external work done by the heart, increasing the rate of coronary flow with erythritol tetranitrate or by the use of a strophanthus preparation (ouabain).

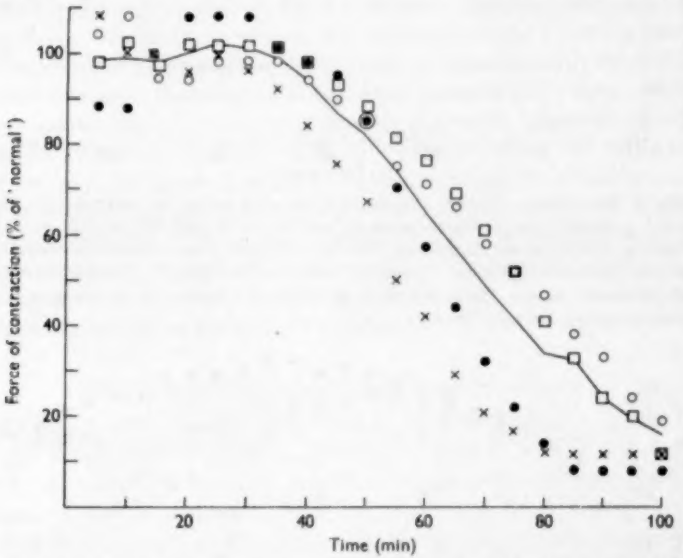



**Fig. 3.** The pattern of contractility changes in hearts perfused with Krebs-bicarbonate medium. The amplitude is recorded as the percentage of the mean recorded amplitude (measured at 5 min intervals over the first 30 min). The different symbols show the time course for four individual hearts. The line is the mean time course of eight hearts, including the four shown individually.

However, bovine plasma albumin (Fraction V, Armour Laboratories) had a marked stabilizing effect (Fig. 4). This effect persisted after dialysis of the protein against water or the bicarbonate medium. Increase in the concentration of albumin up to 2 g/100 ml. was of no additional benefit. Nor was this effect specific to serum albumin: bovine $\gamma$-globulin was even more effective, though its use was limited by its ready denaturation during perfusion. The beneficial effect could be produced more conveniently by dialysed bovine serum (Fig. 4) and here again increase in the concentration of serum above 2 ml./100 ml. produced no definite increase in the effect.

The commencement of the failure of the heart at about 75–85 min in the presence of the serum proteins was found to be partly due to lack of nutrients. When glucose and insulin (200 mg and 500 m-u./100 ml.) were

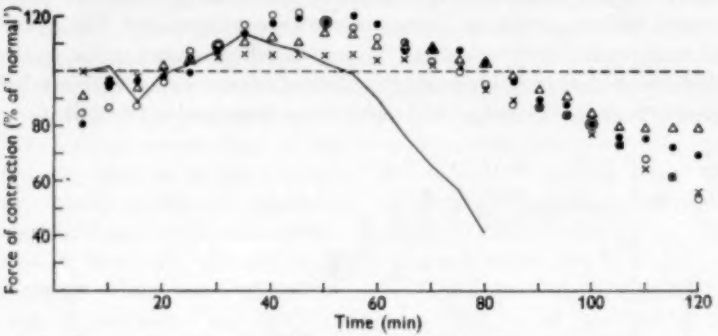



Fig. 4. The effects of serum albumin and dialysed serum on contractility. The basal perfusate was Krebs-bicarbonate medium with half the normal concentrations of calcium and magnesium. The line shows the time course of contractility in the absence of protein. ×, bovine albumin, fraction V (500 mg/100 ml.); ○, dialysed serum (2 ml./100 ml.); ●, dialysed serum (4 ml./100 ml.); △, dialysed serum (16 ml./100 ml.).

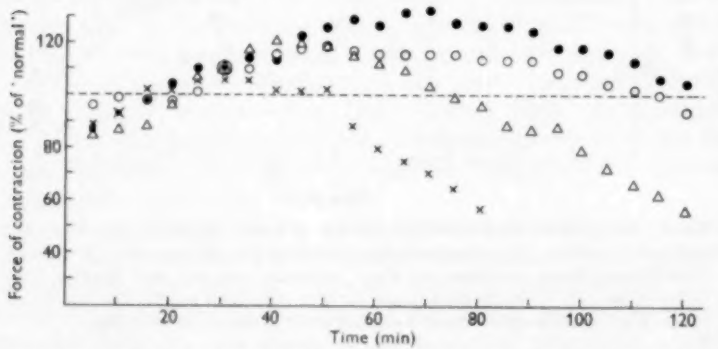



Fig. 5. The effect of nutrients and dialysed serum on contractility. The basal perfusate was the same as in Fig. 4. ×, pyruvate (2 mM); △, dialysed serum (2 ml./100 ml.); ○, dialysed serum (2 ml./100 ml.) + glucose (11 mM) + insulin (5 m-u./ml.); ●, dialysed serum (2 ml./100 ml.) + pyruvate (2 mM).

added to the perfusate as well as dialysed serum (2 ml./100 ml.), the duration of normal contractility was extended to about 115 min (Fig. 5). As glucose would interfere with the uptake of other sugars by the cells, it was desirable to use a different nutrient. Pyruvate was found to be suitable. Thus a modified Krebs-bicarbonate medium containing half the

original concentrations of calcium and magnesium, dialysed bovine serum (2 ml./100 ml.) and sodium pyruvate (2 mM) was found to provide a perfused heart preparation with stable contractility for about 2 hr.

*The source of the effect of protein*

In view of the effectiveness of the different plasma protein preparations, it seemed possible that the effect was due to some non-protein substance bound to the protein. Removal of most of the protein from serum by heat coagulation (10 min at 100° C) did not result in the disappearance of its effectiveness. Similarly, a protein-free solution of the active substance could be obtained by extraction of the serum with methanol or chloroform–methanol mixture. However, the success of extraction by these methods varied considerably; some extracts were undoubtedly active while others prepared by the same methods were not. No preparation was taken to be active unless it produced essentially similar prolongation of the period of full contractility in a set of four hearts. Thus the efficacy of the methods of extraction seemed to depend on factors which were not readily apparent. This problem was not pursued further, because the primary object of this investigation was to obtain a stable perfused heart preparation suitable for the study of cell permeability to sugars.

*The permeability properties of the perfused heart*

Preliminary experiments had shown that L-arabinose penetrated relatively slowly into the perfused heart and that its rate of penetration could be markedly increased by insulin, in agreement with the findings of Goldstein, Henry, Huddlestun & Levine (1953) and Park (1953). It was chosen on these grounds as a convenient indicator of permeability. Experiments to determine the effect of the duration of perfusion on permeability were made by perfusing the heart for a variable period with arabinose-free perfusate and then transferring the heart to a second apparatus containing perfusate identical in all respects except that it contained 30 mM L-arabinose and, in most cases, raffinose (30 mM); perfusion was continued in this apparatus for 15 min. The uptake of sugar by the heart cells in the 15 min of perfusion was then estimated from analysis of the heart extracts and the perfusate, as described under Methods. When the uptake of sugar after different periods of pre-perfusion with sugar-free media was estimated in this way, the results depicted in Fig. 6 were obtained.

It is seen that the permeability of the heart fell sharply in the first 30 min, whether or not the perfusate contained serum protein. In the absence of serum protein the permeability then began to rise again immediately, whereas in its presence there was no appreciable rise in

permeability until the pre-perfusion time exceeded 90 min, i.e. until the total perfusion time exceeded 150 min.

*The effect of pyruvate and dialysed serum on permeability*

It is seen in Fig. 6 that in the earlier periods of perfusion with the modified medium the sugar penetration was consistently lower than the values for the corresponding periods when the plain perfusate was used. In order to determine if this effect was quantitatively related to the additives in the improved medium, the concentrations of dialysed serum and pyruvate were doubled (to 4 ml./100 ml. and 4 mM respectively). The results of the two series are compared in Table 1. There was no consistent difference between them.

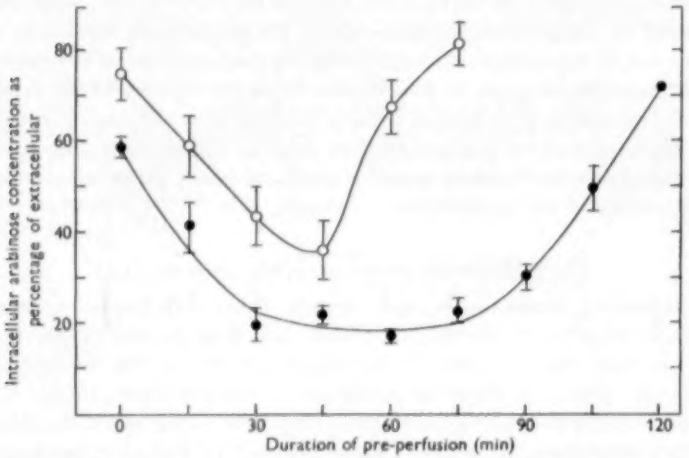



Fig. 6. Changes in heart cell permeability to L-arabinose. Hearts were pre-perfused with sugar-free perfusate for the time shown on the abscissa and then perfused for 15 min with similar perfusate containing 30 mM L-arabinose. The perfusate was modified Krebs-bicarbonate medium (●) or the same with added dialysed serum (2 ml./100 ml.) and pyruvate (2 mM) (○). The vertical bars represent ± the standard error of the mean of 5–8 measurements.

*The initial fall in permeability*

Since a possible cause of this fall might be the disappearance of insulin from the heart, perfusions were made of hearts from animals which had received anti-insulin serum (Wright, 1959) 1 hr before the removal of the heart. These hearts were perfused immediately on removal (i.e. without pre-perfusion) with the modified medium containing L-arabinose (30 mM) for 15 min. In four experiments the intracellular arabinose concentration

was 11·20 % ± 3·66 (4) of the extracellular concentration, which is not significantly different from the value of 19·61 % ± 3·50 (7) found in hearts from normal animals after pre-perfusion for 30 min, but very significantly less than the value of 74·70 ± 5·70 (4) found in normal hearts perfused for 15 min immediately after removal from the animal.

TABLE 1. Percentage penetration of L-arabinose

| Duration of pre-perfusion (min) | Series I | Series II |
|---|---|---|
| 0 | 57·4 + 3·6 (3) | 60·5 (2) |
| 15 | 38·5 + 1·8 (3) | 45·0 (2) |
| 30 | 19·0 + 4·3 (3) | 20·1 + 5·8 (4) |
| 45 | 21·7 + 2·5 (4) | 22·1 + 3·0 (4) |
| 60 | 19·6 + 1·1 (4) | 14·7 + 1·9 (4) |
| 75 | 22·1 + 4·6 (3) | 23·0 + 4·0 (4) |
| 90 | 31·9 + 4·1 (3) | 29·4 + 4·1 (5) |
| 105 | 52·0 + 2·9 (4) | 48·0 + 5·7 (4) |

In Series I the perfusate contains 2 mM pyruvate and 2 ml. ox serum/100 ml. In Series II it contains 4 mM pyruvate and 4 ml. ox serum/100 ml.

*Response to insulin*

In order to determine if the pre-perfused preparation retained the normal permeability responses of the heart cells, the effect of insulin on it was studied. After pre-perfusion with sugar-free modified medium, the hearts were perfused for 15 min with the modified medium containing L-arabinose (30 mM) and insulin (1 m-u./ml. = 0·045 μg/ml.). After 30 min of pre-perfusion insulin raised the sugar penetration from 19·61 % ± 3·50 (7) to 60·06 % ± 5·26 (4). After 75 min of pre-perfusion the uptake of sugar in 15 min in the presence of insulin was 58·81 % ± 2·21 (4). It is clear that the pre-perfused preparation can respond to insulin during the state of basal steady permeability to L-arabinose and that this response does not vary with the duration of pre-perfusion.

*Chloride content of the hearts*

Table 2 gives the intracellular chloride concentration in perfused hearts as a percentage of the chloride concentration in the perfusate. The results indicate a high and constant entry of chloride into the hearts during perfusion irrespective of the composition of the perfusate or the duration of perfusion.

*Intracellular water content of the heart*

Table 3 shows the mean value of intracellular water content of series of hearts perfused for different times with the two perfusates. The figures indicate that the gross composition of the cells is uninfluenced by either of the variables. Three series of experiments with insulin and modified

perfusate not otherwise reported in this paper gave the values of 2·490 ± 0·018 (19) for 0·1 m-u. insulin/ml., 2·424 ± 0·031 (10) for 0·3 m-u./ml. and 2·428 ± 0·021 (5) for 2·0 m-u./ml. The over-all mean of forty-two determinations on hearts perfused with modified perfusate without insulin was 2·441, and the over-all mean of thirty-four hearts perfused with modified medium plus insulin was 2·444. It is therefore clear that insulin has no effect on the water content of the cells.

TABLE 2. Percentage penetration of chloride into intracellular water

| Duration of perfusion (min) | Saline perfusate | Modified perfusate |
|---|---|---|
| 15 | 18·8 + 5·3 (4) | 22·6 + 1·3 (4) |
| 30 | 21·7 + 1·6 (5) | 18·9 + 4·3 (4) |
| 45 | 24·0 + 2·6 (4) | 20·9 + 1·7 (6) |
| 60 | 22·0 + 2·6 (4) | 23·3 + 1·6 (5) |
| 75 | 25·3 + 0·2 (5) | 24·5 + 1·7 (8) |
| 90 | 26·6 + 1·7 (4) | 21·8 + 1·4 (5) |

TABLE 3. Cell water content of perfused hearts (ml./g dry wt.)

| Total duration of perfusion (min) | Perfusate | | |
|---|---|---|---|
| | Plain medium | Modified medium | Modified medium + insulin (0·1 m-u./ml.) |
| 15 | 2·525 ± 0·072 (3) | 2·467 ± 0·026 (4) | — |
| 30 | 2·526 ± 0·072 (4) | 2·449 ± 0·037 (4) | — |
| 45 | 2·410 ± 0·052 (3) | 2·424 ± 0·052 (4) | 2·478 ± 0·036 (3) |
| 55 | — | — | 2·467 ± 0·029 (4) |
| 60 | 2·345 ± 0·033 (3) | 2·422 ± 0·024 (8) | — |
| 65 | — | — | 2·534 ± 0·040 (4) |
| 75 | 2·471 ± 0·059 (4) | 2·361 ± 0·035 (4) | 2·457 ± 0·046 (4) |
| 90 | 2·378 ± 0·072 (3) | 2·474 ± 0·050 (5) | 2·513 ± 0·044 (4) |
| 105 | — | 2·400 ± 0·057 (7) | — |
| 120 | — | 2·496 ± 0·081 (6) | — |
| Over-all mean | 2·448 ± 0·029 (20) | 2·441 ± 0·018 (42) | 2·490 ± 0·018 (19) |

Analysis of variance has been performed on some of these figures to study more precisely the effects of time of perfusion on cell water content. No significant effect exists. It has also been shown that when the hearts are classified according to the extent of pentose penetration into their cells there is no detectable relation between extent of penetration and cell water content.

## DISCUSSION

### *The failure of perfused hearts*

There appear to be only two reports of stable contractility in hearts perfused with saline media (Chenoweth & Koelle, 1946; Nakamura, Saunders, Webb, Lawson & Thienes, 1949). The majority of workers have found that the perfused heart undergoes rapid deterioration, especially

when the medium contains salts only. In the present experiments the failure of the perfused hearts seems to occur in two stages. First, there occur hypodynamic changes which are not related to the lack of nutrients and which can be delayed by the addition of serum proteins to the perfusate. When the heart is enabled to contract normally for a longer period by these means, a hypodynamic state appears after 70–80 min of perfusion, which can be made good by glucose or pyruvate and, presumably, by other substrates. However, the hearts do eventually fail at the end of 2 hr. On the basis of his experiments on frog hearts, Clark (1913–14) suggested that a lipid substance was removed from the heart during perfusion and that the sustaining effect of blood was due to the lipids in it. Recently Hajdu, Weiss & Titus (1957) have shown that one of the substances in certain tissue extracts which had a similar effect on the frog heart was $\beta$-palmitoyl lysolecithin. It is probable that the stabilizing effect of serum and the serum proteins in the present experiments was also due to serum lipids. However, it is possible that some serum proteins themselves were also involved in this effect, since Hajdu & Leonard (1958) have demonstrated, in the blood of dogs, rabbits, monkeys and hypertensive human patients, a protein system which has a cardiotonic action.

It is known that the anti-insulin serum nullifies the action of insulin both *in vivo* and *in vitro* by a reaction of the antigen-antibody type (Moloney & Coval, 1955; Wright, 1959). Therefore it can be taken that the low permeability of the hearts from animals pre-treated with anti-insulin serum is the basic permeability of insulin-free hearts and that the high sugar uptake in normal freshly isolated hearts is due to the presence of insulin activity. This residual insulin activity disappears only after perfusion for about 30 min. At the end of this period the sugar permeability is of the same order as that of the hearts pre-treated with the anti-insulin serum. It is unlikely that loss of sensitivity to insulin plays any significant part in the effect of pre-perfusion, because the cells do respond to insulin in a striking manner even after perfusion for 75 min. It is not possible to decide from the present results whether the residual insulin effect is due to insulin present in the extracellular space, as suggested by the experiments of Bleehen & Fisher (1954), or due to insulin bound to the cells (Stadie, Haugaard & Vaughan, 1952).

The lability of sugar permeability in hearts perfused with saline medium is a factor which has not hitherto received attention in sugar permeability studies. Under the most favourable present conditions this lability restricts the period for study of the kinetics of permeation to the 60–75 min period of stable permeability that follows pre-perfusion for 30 min with medium containing serum protein.

It is seen in Fig. 6 that the difference in the permeability of hearts perfused with plain and modified media persists even at 30 min, at which time presumably the residual insulin activity in the heart has completely disappeared. This suggests that low permeability in the presence of serum is not due to some anti-insulin factor. There seems to be no quantitative relation between the sugar permeability and the additives in the modified medium, since an increase in the concentrations of serum and pyruvate is without effect on sugar permeability.

The marked increase in sugar permeability with longer periods of perfusion appears to be related in some way to the onset of failure of the heart. But it is not profitable to speculate on the significance of this relation, since it is not exact enough. In hearts perfused with the saline medium the beginning of the decline of amplitude of beat occurs at about 50–60 min, whereas the permeability rise is not seen until the period 60–75 min is reached. In hearts perfused with medium containing serum protein and pyruvate the amplitude of the beat is declining between 90 and 100 min, though it is still some 20% above the 'normal' level, i.e. the mean amplitude of the first 30 min, and the amplitude does not fall to the 'normal' level until approximately 120 min. With this medium the permeability rise is first seen in the period 90–105 min.

Thus the first signs of decline in amplitude of beat occur before increase in permeability in the saline perfusions, and coincide with changes in permeability in the serum–saline perfusions. Decline in amplitude below 'normal' level precedes increase in permeability in saline perfusions but occurs much later than increase in permeability in serum–saline perfusions. On either basis it is difficult to relate the change in permeability closely with the change in amplitude of beat, though there is a presumption of relation in the fact that the modifications of the perfusate which we have described result in the maintenance of both these properties at a much steadier level than is shown in saline perfusions.

The figures show that there is a consistently high intracellular chloride content of these hearts unrelated to the potassium content and to the force of the contraction. The extent of penetration of chloride observed in these experiments is independent of the time of perfusion, the nature of the perfusate, the state of sugar permeability, or the presence or absence of insulin. Although, therefore, the hearts used were manifestly abnormal, the abnormality seems to have no bearing on the mechanism of sugar penetration and the effect of insulin on it. Creese (1954) found that isolated rat diaphragm took up a considerable amount of sodium, even though the potassium loss was small and the membrane potential was nearly normal (Creese, Scholes & Whalen, 1958). He did not measure the chloride content of his preparation; but his findings together with the present observations

suggest the possibility that there is a movement of sodium chloride into isolated tissues suspended in saline media.

The observations of the extreme constancy of the ratio of intracellular water to heart dry weight means that we can dispense with the direct determination of extracellular water of the heart. Determination of wet weight and dry weight provide all the data necessary for the determination of intracellular and extracellular water, the intracellular water content being 2·45 times the dry weight. The extracellular water is then the difference between this and total water.

## SUMMARY

1. In hearts perfused with Krebs-bicarbonate medium containing half the original concentrations of calcium and magnesium the force of contraction begins to fall after about 55 min of perfusion.

2. This deterioration cannot be delayed by provision of nutrients or reduction in the external work of the heart.

3. Bovine plasma albumin and $\gamma$-globulin, dialysed ox serum, and some extracts of these help to maintain normal contractility for about 70–80 min.

4. If nutrients are also added, the hearts continue to contract with normal force for about 2 hr.

5. The permeability to L-arabinose of hearts perfused with plain Krebs-bicarbonate medium and with the modified medium decreases steadily over the first 30 min of perfusion. This has been shown to be due to the disappearance of residual insulin activity.

6. The mechanical failure of the heart is approximately associated with a large increase in sugar permeability. This occurs in the hearts perfused with plain saline medium soon after the basal permeability is reached, whereas in the hearts perfused with the modified medium there is a period of about 60–75 min of stable basal permeability before the increase in permeability appears. This period can be used for reliable studies of some aspects of sugar permeability.

I wish to record my grateful appreciation of the encouragement and guidance of Professor R. B. Fisher during this investigation and of the kind gift of anti-insulin serum by Dr P. H. Wright.

## REFERENCES

Bleehen, N. M. & Fisher, R. B. (1954). The action of insulin in the isolated rat heart. *J. Physiol.* **123**, 260–276.

Boyle, P. J. & Conway, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1–63.

Bronk, M. S. & Fisher, R. B. (1957). The interaction of growth hormone and insulin in the perfused rat heart. *J. Physiol.* **136**, 435–448.

CHENOWETH, M. B. & KOELLE, E. S. (1946). An isolated heart perfusion system adapted to the determination of nongaseous metabolites. *J. Lab. clin. Med.* **31**, 600–608.

CLARK, A. J. (1913–14). The action of ions and lipoids upon the frog's heart. *J. Physiol.* **47**, 66–107.

CREESE, R. (1954). Measurement of cation fluxes in rat diaphragm. *Proc. Roy. Soc.* B, **142**, 497–513.

CREESE, R., SCHOLES, N. W. & WHALEN, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

FISHER, R. B. & LINDSAY, D. B. (1956). The action of insulin on the penetration of sugars into the perfused heart. *J. Physiol.* **131**, 526–541.

FUJITA, A. & IWATAKE, D. (1931). Bestimmung des echten Blutzuckers ohne Hefe. *Biochem. Z.* **242**, 43–60.

GOLDSTEIN, M. S., HENRY, W. L., HUDDLESTUN, B. & LEVINE, R. (1953). Action of insulin on transfer of sugars across cell barriers: common chemical configuration of substances responsive to action of the hormone. *Amer. J. Physiol.* **173**, 207–211.

GREENE, C. H. & POWER, M. H. (1931). The distribution of electrolytes between serum and the *in vivo* dialysate. *J. biol. Chem.* **91**, 183–202.

HADJU, S. & LEONARD, E. (1958). 'A serum protein system affecting contractility of the frog heart present in increased amounts in patients with essential hypertension. *Circulation Res.* **6**, 740–750.

HADJU, S., WEISS, H. & TITUS, E. (1957). The isolation of a cardiac active principle from mammalian tissue. *J. Pharmacol.* **120**, 99–113.

HELMREICH, E. & CORI, C. (1957). Studies of tissue permeability. II. The distribution of pentoses between plasma and muscle. *J. biol. Chem.* **224**, 663–679.

KREBS, H. A. & HENSELEIT, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyl. Z.* **210**, 33–66.

MOLONEY, P. J. & COVAL, M. (1955). Antigenicity of insulin: diabetes induced by specific antibodies. *Biochem. J.* **59**, 179–185.

NAKAMURA, K., SAUNDERS, P. R., WEBB, J. L., LAWSON, H. C. & THIENES, C. H. (1949). Metabolism of the heart in relation to drug action. IV. Effects of various substrates upon the isolated perfused rat heart. *Amer. J. Physiol.* **158**, 269–278.

PARK, C. R. (1953). The action of insulin on glucose uptake by muscle. *J. clin. Invest.* **32**, 593.

ROE, J. H. & RICE, E. W. (1948). A photometric method for the determination of free pentoses in animal tissues. *J. biol. Chem.* **173**, 507–512.

SENDROY, J. (1937). Microdetermination of chloride in biological fluids, with solid silver iodate. *J. biol. Chem.* **120**, 405–417.

STADIE, W. C., HAUGAARD, N. & VAUGHAN, M. (1952). Insulin binding with isotopically labelled insulin. *J. biol. Chem.* **199**, 729–739.

WRIGHT, P. H. (1959). The effect of insulin antibodies on glucose uptake by the isolated rat diaphragm. *Biochem. J.* **71**, 633–638.

*J. Physiol.* (1961), **158**, *pp.* 73–85
*With* 3 *text-figures*
*Printed in Great Britain*



# THE MECHANISM OF THE UPTAKE OF SUGARS BY THE RAT HEART AND THE ACTION OF INSULIN ON THIS MECHANISM

BY R. B. FISHER* AND P. ZACHARIAH†
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

The possibility that the action of insulin involves the permeability of muscle cells to sugars (reviewed by Levine & Goldstein, 1955) has led to a number of investigations on the kinetics of sugar uptake by muscles. Some of these have been interpreted as showing that sugar uptake follows first-order kinetics (Kipnis & Cori, 1957; Resnick & Hechter, 1957). This is in disagreement with the observations on erythrocytes (reviewed by Bowyer, 1957) and with some studies on muscle preparations (reviewed by Park, Reinwein, Henderson, Cadenas & Morgan, 1959). This paper presents some findings on the time course of the uptake of two non-metabolized sugars by a perfused rat heart preparation in which special care has been taken to ensure adequacy of perfusion, stability of sugar permeability and freedom from endogenous insulin action (Zachariah, 1961). It is shown that these results are consistent with a carrier-type mechanism for the transport of sugars. The effects of insulin on the kinetics of the carrier mechanism suggest new interpretations of the nature of insulin action.

## *Theoretical*

Two characteristics of sugar uptake processes that need to be explained by any model of the transport mechanism are of special significance in the choice of a kinetic model: (i) The uptakes of certain sugars apparently reach equilibrium even when a considerable concentration gradient may still be present across the cell membrane. (ii) The relation between the rate of sugar uptake and external sugar concentration is, in the case of many sugars, not linear but of the Michaelis–Menten type. It has been repeatedly suggested that (i) is due to the presence of intracellular barriers to sugar entry in the absence of insulin (Kipnis & Cori, 1957; Morgan, Randle & Regan, 1959). But the observations that the 'volume of distribution' of the sugar is inversely related to its external concentration, as

* Present address: Department of Biochemistry, University of Edinburgh.
† Present address: Department of Physiology, Christian Medical College, Vellore, S. India.

can be deduced from the observations of Wick & Drury (1953), Drury & Wick (1954) and Morgan & Park (1958) and that the volumes of distribution of different sugars under identical conditions vary widely (Helmreich & Cori, 1957; Norman, Menozzi, Reid, Lester & Hechter, 1959) make it difficult to maintain a simple concept of an intracellular barrier responsive to insulin. The second characteristic points to a mechanism other than simple diffusion.

A simple concept which can explain these two phenomena as well as many other characteristics of sugar uptake is the carrier hypothesis (Widdas, 1952, 1954). In a simplified version of this model (Bowyer, 1957), the sugar can be described as traversing the membrane in combination with a component of the membrane, the carrier. The carrier may be assumed to move across the membrane by a process equivalent to diffusion at a rate independent of whether it is in combination with the sugar or not. The velocity constants for the formation and dissociation of the sugar–carrier complex may be taken to be the same on the two surfaces of the membrane. In such a model the rate of transport of the sugar would depend primarily on the difference between the concentrations of the sugar–carrier complex at the two boundaries of the membrane.

TABLE 1. Effect of alterations in the equilibrium constant of the dissociation of sugar-carrier complex on the rate of sugar uptake

| Apparent equilibrium constant '$M$' (mM) | Concn. of sugar-carrier complex | | |
|---|---|---|---|
| | Outer surface $SC_o$ (mM) | Inner surface $SC_i$ (mM) | $SC_o - SC_i$ (mM) |
| 0·1 | 0·9967 | 0·8571 | 0·1396 |
| 1·0 | 0·9677 | 0·3750 | 0·5927 |
| 10·0 | 0·7500 | 0·0566 | 0·6934 |
| 100·0 | 0·2308 | 0·0060 | 0·2248 |

Sugar concentration at the outer surface, 30 mM; sugar concentration at the inner surface, 0·6 mM. Total amount of carrier at each surface is assumed to be equal to 1 mM. $SC_o$ = fraction of available carrier combined with sugar at outer membrane surface; $SC_i$ = fraction of available carrier combined with sugar at inner membrane surface.

A low concentration gradient of the sugar–carrier complex across the membrane can result from a very low or a very high affinity of the carrier for the sugar. This is best illustrated by considering an example. Taking it that the carrier is equally distributed between outer and inner parts of the membrane and that the fractional saturation of carrier is given by the ratio $S/(S+K)$, where $S$ = sugar concn., and $K$ = equilibrium constant of the sugar–carrier reaction, then the state of affairs at a given intracellular concentration can be illustrated by the example in Table 1.

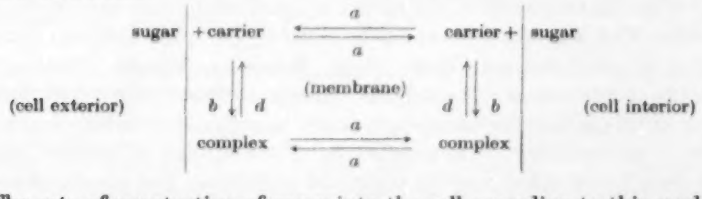
It will be clear from this Table that at intracellular concentrations which are low relatively to the external one, the carrier at the inner border of

the membrane may be so nearly saturated, provided that the equilibrium constant is low enough, that further sugar penetration must be very slow. Increase in equilibrium constant, though it reduces the fractional saturation at the outer border of the membrane appreciably, has a much more marked effect on saturation at the inner border, and the sugar–carrier complex concentration gradient is markedly increased. When the equilibrium constant is still further increased the fractional saturation of carrier at the outer border is further decreased markedly and this decrease can more than offset further diminution in saturation at the inner border, thus diminishing the sugar–carrier complex concentration gradient.

If sugar penetration conforms to the carrier model, insulin could increase penetration in one of four ways: by (*a*) increasing the amount of available carrier, (*b*) increasing the mobility of the carrier–complex in the membrane, (*c*) increasing affinity of carrier for sugar if the carrier has a low affinity, (*d*) decreasing affinity of carrier for sugar if the carrier has a very high affinity.

Test of the applicability of the carrier hypothesis and exploration of the mode of action of insulin can therefore be made by following the time course of penetration of sugars, and by endeavouring to find values for the parameters of penetration which will throw light on any changes caused by insulin in carrier concentration and affinity.

The general nature of the model proposed by Widdas is shown in the following scheme:




The rate of penetration of sugar into the cell according to this model is given by Bowyer (1957) as:

$$\text{Transfer rate} = \frac{Kad}{2b} \cdot \frac{C_1 - C_2}{C_1C_2 + \left(\frac{a+d}{b}\right)(C_1 + C_2) + \left(\frac{a+d}{b}\right)^2 \frac{a^2}{b^2}}, \qquad \text{(i)}$$

where $K$ = the sum of the concentrations of carrier at the two surfaces,
$C_1$ = concentration of sugar at the external surface,
$C_2$ = concentration of sugar at the internal surface.
$a$ = transfer constant of carrier (free or combined) in the membrane,
$b$ = velocity constant of sugar–carrier complex formation, and
$d$ = velocity constant of sugar–carrier complex dissociation.

This can be rearranged and integrated if it is assumed that $a^2/b^2$ is negligible. In fact it can be shown that in our conditions, provided that $a$ is appreciably smaller than $d$, no significant error is introduced by neglecting this term. Since $d$ is the velocity constant of dissociation of the complex and $a$ that of transfer across the membrane, such a relation of the two constants seems likely.

When the external sugar concentration is constant and equal to $C_1$ and the initial internal concentration is zero, the integrated equation is:

$$(C_1+M)^2 \ln \frac{1}{1-f} - C_1(C_1+M)f = Nt, \qquad \text{(ii)}$$

$$M = \frac{a+d}{b}, \quad N = \frac{Kad}{2b} \quad \text{and} \quad f = C_2/C_1.$$

Thus $M$ is the apparent dissociation constant of the sugar–carrier complex and $N$ may be called a membrane constant. When $C_1$, $M$ and $N$ are constant, changes in $t$ will produce proportionate changes in the left-hand side of equation (ii). Thus if $f$ values for the same concentration of sugar are determined at different values of $t$ (i.e. the time course of sugar uptake), it is possible to estimate the values of $M$ and $N$.

One interesting aspect of the carrier hypothesis is that it accounts for a phenomenon which is difficult to explain in any other way, namely that the movement of a sugar in one direction can be influenced by the movement of another sugar using the same transport mechanism in the opposite direction. This was first pointed out by Widdas (1952) and has been demonstrated in erythrocytes (Park, Post, Kalaman, Wright, Johnson & Morgan, 1956), and in the rat heart (Morgan & Park, 1958). One implication of this is that the same sugar under appropriate conditions can act either as an inhibitor or as accelerator of the uptake of another sugar. This paradoxical effect may be explained as follows: The uptake of sugar by the carrier mechanism can be described as the algebraic sum of the independent processes of influx and efflux of the sugar. Thus any factor which tends to inhibit the influx more than the efflux would tend to produce a reduction in the net uptake of sugar. This is the effect observed when a mixture of a test sugar and a competing sugar is added to the external medium. On the other hand, any factor which tends to produce a preferential inhibition of efflux would tend to cause an acceleration of the net uptake of sugar. Such an effect can be produced by a competing sugar, if it is present in about the same or greater concentration inside the cell as on the outside before the test sugar is added to the medium. In such a situation, in the early stages of the uptake of the test sugar its efflux will be inhibited much more than its influx because the ratio of the inhibitor

to the test sugar is much greater on the inside than on the outside. A demonstration of this effect would add to the evidence in favour of the carrier hypothesis.

## METHODS

The technique of perfusion of the heart, the modified Krebs-bicarbonate perfusion medium containing serum protein, and the method of estimating the sugars have been described elsewhere (Zachariah, 1961).

All hearts were perfused for 30 min with a medium lacking the sugar to be studied and then transferred to a second apparatus filled with medium containing this sugar in a standard concentration (30 mM). Hearts were then perfused for 15, 25, 35, 45 or 60 min, and fractional penetration into the cell water was then determined.

The preliminary period of perfusion with the sugar-free medium is referred to as 'pre-perfusion' and the period of subsequent perfusion with the sugar-containing medium as 'perfusion'.

Insulin (crystalline, British Drug Houses, Batch No. 2189), when used, was present in the media for both perfusion and pre-perfusion in a concentration of 0·2 m-u./ml.

It has been shown that under the conditions of the present experiments the intracellular water content of the cell is constant and has the mean value of 2·450 ml./g dry weight (Zachariah, 1961). On this basis the cell-water content, extracellular space and fractional penetration of cell water by sugar were calculated from the dry weight of the tissue by methods described previously.

## RESULTS

### *Time course of permeation of the heart by pentoses*

The number of determinations made for the estimation of extent of permeation at any one time varied between 4 and 8. The means and standard errors are given in Table 2, and the closest-fit curves of the form of equation (ii) are shown in Figs. 1 and 2.

TABLE 2. Time course of penetration of L-arabinose and D-xylose into the intracellular water of the perfused rat heart in the presence and absence of insulin (0·2 m-u./ml.)

| Length of perfusion (min) | L-arabinose | | D-xylose | |
|---|---|---|---|---|
| | No insulin | Insulin | No insulin | Insulin |
| 15 | 17·04 ± 2·91 (6) | 38·59 ± 5·03 (5) | 22·43 ± 2·14 (5) | 46·12 ± 5·49 (4) |
| 25 | 16·25 ± 1·90 (8) | 45·99 ± 4·01 (6) | 27·42 ± 2·53 (5) | 57·46 ± 3·00 (4) |
| 35 | 22·33 ± 3·72 (7) | 59·34 ± 7·92 (5) | 30·91 ± 2·70 (5) | 73·33 ± 2·80 (5) |
| 45 | 25·31 ± 2·61 (7) | 70·44 ± 5·47 (5) | 34·23 ± 3·76 (5) | — |
| 60 | 25·58 ± 2·96 (8) | 73·80 ± 5·57 (6) | 40·24 ± 3·61 (5) | 80·00 ± 4·17 (5) |

The figures give the intracellular concentration as percentage of the extracellular concentration (30 mM) with the standard errors of the mean and the numbers of observations in each set.

The values of the constants $M$ and $N$ (eqn. ii) corresponding to these fitted curves are given in Table 3. It will be seen that in the absence of insulin the values of $M$, the half-saturation concentration, are very low compared with the concentration of pentose used (30 mM), and that in the presence of insulin they are much higher, i.e. there is apparently a fall in the affinity of the carrier for the pentose.

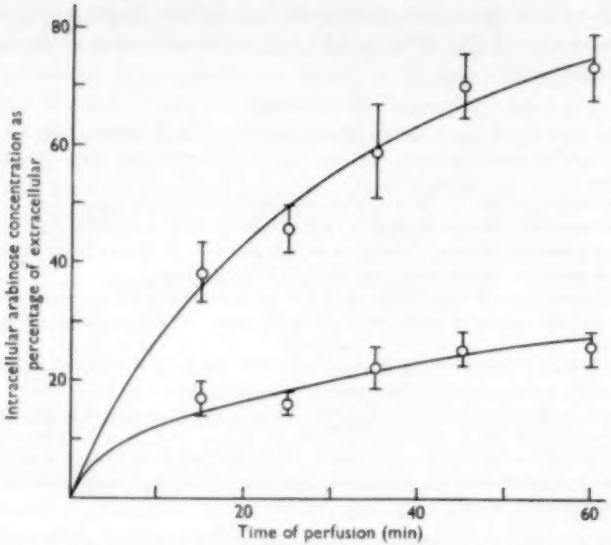



Fig. 1. The time course of penetration of L-arabinose (30 mM) into the cells of the heart. The vertical bars represent ± the standard error of the mean. The upper curve refers to the insulin experiments. The insulin concentration was 0·2 m-u./ml.

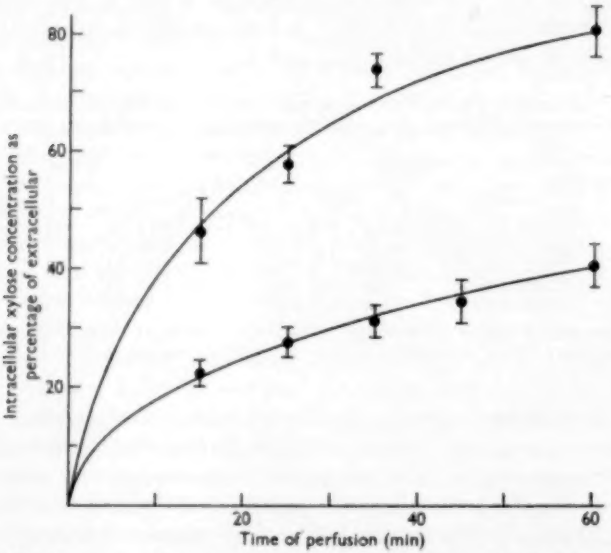



Fig. 2. The time course of penetration of D-xylose (30 mM) into the cells of the heart. The vertical bars represent ± S.E. of the mean. The upper curve refers to the insulin experiments. The insulin concentration was 0·2 m-u./ml.

*Effect of glucose on the penetration of pentoses into the heart.* Hearts were perfused for 30 min with protein–pyruvate medium and then perfused for 35 min with similar medium with added pentose (30 mM) and glucose (10 mM). The effect of this treatment on the percentage permeation of the hearts by pentose is shown in Table 4. There is no doubt that glucose exerts a profound inhibitory effect on permeation.

TABLE 3. Estimated values of '$M$' and '$N$'

| | | L-arabinose | D-xylose |
|---|---|---|---|
| No insulin | $M$ (mM) | 0·06 | 0·21 |
| | $N$ (mM$^2$.min$^{-1}$) | 0·71 | 1·71 |
| | $N/M$ (min.mM$^{-1}$) | 11·8 | 8·1 |
| Insulin | $M$ (mM) | 25·0 | 6·6 |
| (0·2 m-u./ml.) | $N$ (mM$^2$.min$^{-1}$) | 50·3 | 22·8 |
| | $N/M$ (min.mM$^{-1}$) | 2·0 | 3·5 |

These values provide the best fit of the observations on the time course of sugar uptake to the descriptive equation for transport by the carrier mechanism given as equation (ii) on p. .

TABLE 4. Inhibition of pentose uptake by glucose

| Pentose (30 mM) | Insulin (0·2 m-u./ml.) | Percentage penetration of cell water by pentose in 35 min* Without glucose ($A$) | With glucose (10 mM) ($B$) | $A-B$ | $100\frac{A-B}{A}$ |
|---|---|---|---|---|---|
| L-Arabinose | Nil | 22·33 ± 3·72 (7) | 8·95 ± 1·72 (4) | 13·38 | 59·9 |
| | + | 59·34 ± 7·92 (5) | 42·96 ± 7·19 (5) | 16·38 | 27·6 |
| D-Xylose | Nil | 30·91 ± 2·70 (5) | 23·63 ± 3·01 (6) | 7·28 | 23·6 |
| | + | 73·33 + 2·80 (5) | 60·83 + 4·69 (5) | 12·50 | 17·0 |

* Mean ± S.E. of mean (no. of observations).

*Effect of galactose on permeation.* One series of experiments with L-arabinose was made in the same way as the glucose experiments, using 10 mM galactose. The mean percentage penetration of the intracellular water in 35 min was 18·44 ± 2·82 (5) as compared with the corresponding figure without galactose (Table 1) of 22·33 ± 3·72 (7).

In a second series of experiments the hearts were pre-perfused with 10 mM galactose and then perfused for 35 min with 30 mM L-arabinose and 10 mM galactose. The mean percentage penetration of the pentose was 24·62 ± 3·57 (5).

There is here an indication that galactose can either accelerate or slow down penetration of the pentose, depending on the way in which it is presented to the heart.

## DISCUSSION

The time course of sugar penetration found in these experiments does not conform to first-order kinetics. Figure 3 is a plot of ln $1/(1-f)$

against time of the results of Table 2. Although there is an approximately linear relation in all cases, no one of the lines goes through the origin, and the intercepts are far too large to be ascribed to experimental error. For first-order relations the lines must pass through the origin.

It has previously been suggested (Bronk & Fisher, 1957) that the time course of sugar penetration could be accounted for on the hypothesis that uniformly slow diffusion occurred throughout the cell contents. A curve describing this sort of process has been presented by Crank (1956) and it can be shown that the present data do in fact conform to such a curve.

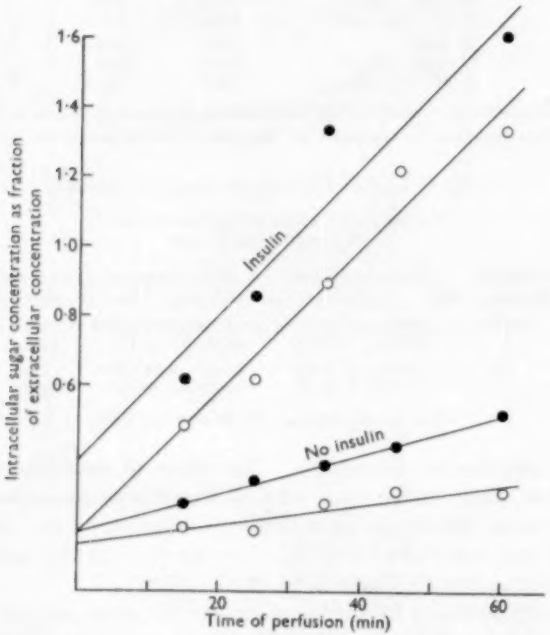



Fig. 3. The lack of correspondence of sugar penetration with first-order kinetics. ○ = L-arabinose; ● = D-xylose.

However, the relationship described in equation (ii) is indistinguishable from the Crank curve in the range of values of $M$ and $N$ applicable to our data.

Discrimination between the slow-diffusion and carrier hypotheses can, however, be effected. The slow-diffusion hypothesis cannot explain the competition between pentoses and glucose or galactose which has been demonstrated in these experiments and in those of Morgan & Park (1958). Further, the experiments in which galactose presented to the outside of

the cell has an inhibitory effect, whilst galactose presented simultaneously to both sides of the cell membrane has an acceleratory effect, suggest strongly that the determinants of permeation are in the cell membrane itself.

On the other hand, all these effects are precisely what is to be expected on the carrier hypothesis. We are therefore of the opinion that the conformity of the observations to equation (ii), together with the competition and acceleration effects, make a *prima facie* case for the carrier hypothesis.

A decision between the hypotheses could be made on an experimental basis. If intracellular concentration is expressed as a fraction of extracellular concentration the Crank curve should be of the same form whatever the concentration of sugar added to the perfusate. On the other hand, as inspection of equation (ii) will show, on the carrier hypothesis, the form of the curve for any given pair of values of $M$ and $N$ will be markedly dependent on the external sugar concentration. Experiments to test the effect of change of concentration of sugar on the time course of penetration are in progress.

*The effects of galactose on pentose penetration*

It will be seen that the two effects of galactose which have been described are of small magnitude, and not very firmly established. A probable reason for this is that the initial favourable state for the observation of these effects would, on the carrier hypothesis, rapidly disappear. If galactose is initially wholly outside the cells it can compete with the pentose only by combining preferentially with carrier, and so will penetrate the cells and reduce the effective affinity for pentose of the carrier at the inner border of the membrane, thereby increasing the concentration difference of pentose-carrier complex across the membrane and offsetting, at least in part, the inhibitory effect of the external galactose. If galactose is initially in equal concentration on both sides of the membrane, i.e. if galactose flux in both directions is equal, the presentation of pentose to the outer border of the cell membrane will inhibit galactose influx and bring about a net loss of galactose from the cell. This sort of effect has already been described by Morgan & Park (1958) for hearts equilibrated with 3-methylglucose and then perfused with glucose and 3-methylglucose. This effect will result in rapid diminution of the initial acceleratory effect of galactose. The finding of conditions in which pentose penetrations of adequate extent for exact measurement can take place in very short times is a prerequisite for the full establishment of the dual effects of a competing sugar.

*The effect of glucose on pentose penetration*

It is not possible to interpret these data in any detail. Since glucose entering the cells will be metabolized in large part, and since the pentose will alter the rate of entry, a complex situation results. The important point is that glucose does inhibit the penetration of the two pentoses studied both in the presence and absence of insulin, and there is therefore ground for supposing that the pentose penetration process is also the process responsible for the penetration of glucose into the cells. The common sensitivity of these processes to insulin suggests that the mode of action of insulin on pentose penetration which we propose to deduce from our observations is also the mode of action of insulin on glucose penetration.

*The significance of the parameters of the carrier equation*

The values of the constants $M$ and $N$ in equation (ii) which correspond to the curves fitted to the data in Fig. 1 are given in Table 3. The constant $M$ is the Michaelis constant of combination of carrier with sugar. It will be seen that, in the absence of insulin, this constant is very much lower than the external sugar concentration used. When insulin is present this constant rises markedly. Thus, if the carrier hypothesis is valid, one effect of insulin must be to lower markedly the affinity of the carrier for the sugar being transported. As has already been pointed out (p. 74), a carrier with very high affinity for its substrate will be inefficient, and its efficiency will be increased by fall of affinity, within limits.

Insulin appears to have this effect, and this effect would in itself be adequate to account for its action. Before discussing further implications of this interpretation of our results, it is necessary to look at the changes brought about by insulin in the second parameter, $N$, of equation (ii).

The parameter $N$ is equal to $Kad/2b$, where $K$ measures the content of carrier in the membrane, and $a$, $b$ and $d$ are the velocity constants respectively of movement of carrier across the membrane, of association of sugar and carrier, and of dissociation of the sugar-carrier complex. The assumption made in the introduction that the term $a^2/b^2$ in equation (i) is negligible is tantamount to the assumption that $a$ is negligible in relation to $d$. This implies that $M = (a+d)/b$ is negligibly different from $d/b$. Thus $N/M$ is negligibly different from $Ka/2$.

It will be seen from Table 3 that insulin increases the value of $N$, but far less so than that of $M$, so that $N/M$ is lower in the presence of insulin than in its absence. Thus insulin seems either to decrease the amount of carrier or to decrease the ease with which carrier crosses the membrane.

Both these effects are physical possibilities if one assumes that insulin

affects the carrier by combining with it. If the carrier-insulin complex is much more efficient than free carrier, then the effect will be that, in the presence of insulin, sugar transport will be virtually completely effected by that fraction of the carrier combined with insulin. The effective value of $K$ would therefore decrease. If the carrier is a small molecule, the value of $a$ could be markedly decreased by combination of carrier with insulin. If the carrier were a large molecule, such an effect would be less likely.

This analysis leads to several consequences susceptible to experimental test. The first is that, if the effective carrier, in the presence of any significant amount of insulin, is the insulin-carrier complex, then the value of $M$ should not change with increasing concentrations of insulin. The second is that, if the proportion of the carrier present as insulin-carrier complex rises as the insulin concentration increases, the value of $N/M$ should increase with increasing amounts of insulin to a maximum. The third is that this maximal value of $N/M$ should be equal to the value in the absence of insulin if the ease of movement of the insulin-carrier complex across the membrane is indistinguishable from that of free carrier, and should be less than it if the complex moves less readily than free carrier.

*General considerations*

It is important to keep in mind that the permeability of one particular kind of cell membrane to a particular species of solute can be altered in different ways. It has been shown in a previous paper (Zachariah, 1961) that in the absence of insulin the permeability of rat heart cells to pentoses rises sharply with time unless an appropriate plasma protein is present in the perfusate. This phenomenon is not susceptible to analysis by the methods used here, since the permeability of such cells is changing continuously. But, since this increase in permeability is brought about by the absence of something from the perfusate, and not by the presence of something, e.g. of insulin, it is presumably of a different nature. This may have a bearing on the claim by Resnick & Hechter (1957) that these are two separable sugar permeation processes in the rat diaphragm. It seems likely therefore that studies of the effect of insulin must be made either in our conditions of stable permeability or in hearts perfused for sufficiently long with protein-free perfusate to attain, if possible, a new stable state of increased permeability.

The point of greatest general importance in this work is that it has become apparent that 'activation' of the permeation mechanism for sugars by insulin can be accounted for by partial 'inhibition' of the carrier. If subsequent work substantiates this proposition, it might prove of value in connexions other than that of reorienting thinking about the nature of insulin action on sugar transport. There seems no reason why

insulin should not combine with carriers in the cell membrane which are specific for solutes other than sugars, and in instances in which the affinities of such carriers for their substrates are low an action of insulin on them similar to that postulated here for the sugar carrier would result in inhibition of penetration of their substrates into the cells.

*The relation between carriers and enzymes*

The essential feature of a carrier is that it should be capable of combining reversibly with its substrate. If this should be the sole requirement, then any enzyme present in the cell membrane should be able to act as a carrier of its substrate or of its competitive inhibitors. It is even conceivable that an enzyme might transport its substrate without appreciable transformation, especially if the substance of the cell membrane formed an unfavourable medium for co-factors of enzymic action.

It may therefore well be the case that there is no fundamental discrepancy between the view that enzymes play a part in permeation and the view that permeation is a passive process involving specific membrane carriers.

SUMMARY

1. Measurements have been made of the time course of penetration of L-arabinose and D-xylose into the perfused rat heart, using the precautions described by Zachariah (1961) to ensure a period of stable permeability.

2. The effect of a submaximal concentration of insulin (0·2 m-u./ml.) on the time course of penetration of the pentoses has been determined.

3. When glucose is added to the perfusate it reduces the rate of penetration of the pentoses.

4. Galactose added to the perfusate at the same time as the pentose reduces the extent of pentose penetration. If the heart is previously equilibrated with galactose, and pentose is then added to the perfusate, the rate of pentose penetration is increased.

5. These findings are shown to be consistent with the hypothesis that the pentose enters the cells in combination with a specific carrier in the cell membrane.

6. The accelerating effect of insulin on pentose penetration is shown to be explicable in terms of a reduction of the affinity of the carrier for the pentose.

REFERENCES

Bowyer, F. (1957). The kinetics of the penetration of non-electrolytes into the mammalian erythrocyte. *Int. Rev. Cytol.* **6**, 469–511.

Bronk, M. S. & Fisher, R. B. (1957). The interaction of growth hormone and insulin in the perfused rat heart. *J. Physiol.* **136**, 435–448.

Crank, J. (1956). *The Mathematics of Diffusion*. Oxford: Clarendon Press.

DRURY, D. R. & WICK, A. N. (1954). Metabolism of mannose by the extrahepatic tissues. *Amer. J. Physiol.* **177**, 535–538.

HELMREICH, E. & CORI, C. F. (1957). Studies of tissue permeability. II. The distribution of pentoses between plasma and muscle. *J. biol. Chem.* **224**, 663–679.

KIPNIS, D. M. & CORI, C. F. (1957). Studies of tissue permeability. III. The effect of insulin on pentose uptake by the diaphragm. *J. biol. Chem.* **224**, 681–693.

LEVINE, R. & GOLDSTEIN, M. S. (1955). V. Mechanism of hormone action. On the mechanism of action of insulin. *Recent Progr. Hormone Res.* **11**, 343–380.

MORGAN, H. E. & PARK, C. R. (1958). Sugar transport across muscle cell membrane. *Fed. Proc.* **17**, 278.

MORGAN, H. E., RANDLE, P. J. & REGAN, D. M. (1959). Regulation of glucose uptake by muscle. 3. The effects of insulin, anoxia, salicylate and 2:4-dinitrophenol on membrane transport and intracellular phosphorylation of glucose in the isolated rat heart. *Biochem. J.* **73**, 573–579.

NORMAN, D., MENOZZI, P., REID, D., LESTER, G. & HECHTER, O. (1959). Action of insulin on sugar permeability in rat diaphragm muscle. *J. gen. Physiol.* **42**, 1277–1299.

PARK, C. R., POST, R. L., KALAMAN, C. F., WRIGHT, J. H., JOHNSON, L. H. & MORGAN, H. E. (1956). The transport of glucose and other sugars across cell membranes and the effect of insulin. *Ciba Foundation Coll. Endocrinology*, **9**, 240–265.

PARK, C. R., REINWEIN, D., HENDERSON, M. J., CADENAS, E. & MORGAN, H. E. (1959). The action of insulin on the transport of glucose through the cell membrane. *Amer. J. Med.* **26**, 674–684.

RESNICK, O. & HECHTER, O. (1957). Studies on the permeability of galactose in muscle cells of the isolated rat diaphragm. *J. biol. Chem.* **224**, 941–54.

WICK, A. N. & DRURY, D. R. (1953). Action of insulin on volume of distribution of galactose in body. *Amer. J. Physiol.* **173**, 229–232.

WIDDAS, W. F. (1952). Inability of diffusion to account for placental glucose transfer in sheep and consideration of kinetics of possible carrier transfer. *J. Physiol.* **118**, 23–39.

WIDDAS, W. F. (1954). Facilitated transfer of hexoses across human erythrocyte membrane. *J. Physiol.* **125**, 163–180.

ZACHARIAH, P. (1961). Contractility and sugar permeability in the perfused rat heart. *J. Physiol.* **158**, 59–72.

*J. Physiol.* (1961), **158**, *pp.* 86–101
*With 9 text-figures*
*Printed in Great Britain*

# THE OXYGEN UPTAKE OF THE PERFUSED RAT HEART

BY R. B. FISHER* AND J. R. WILLIAMSON
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

Since the publication of the papers of Laitinen & Kolthoff (1941*a*, *b*) and Davies & Brink (1942) stationary, vibrating or rotating platinum electrodes have been extensively used for the estimation of the oxygen consumption of surviving tissues and cell suspensions. This paper describes the development of a method of this kind for the determination of the oxygen consumption of the rat heart perfused through the coronary arteries with a saline-bicarbonate medium.

The results show that although the oxygen uptake of the isolated rat heart is three to four times as great as that of rat heart slices, it is feasible to supply considerably more oxygen than the basal oxygen requirement of the heart. The isolated rat heart preparation is shown to be resistant to short periods of complete anoxia, or longer periods of partial anoxia, and it is suggested that survival time after anoxia is affected by the initial glycogen content of the heart. Some characteristics of the oxygen uptake of hearts perfused without added nutrient are described.

## METHODS

### *Principle of the polarographic method*

The rat heart is supplied with a bicarbonate-saline medium which is kept in equilibrium with a gas mixture containing a known partial pressure of $O_2$ with $CO_2$. The oxygen concentration in the medium issuing from the heart is measured polarographically, and the rate of flow of medium through the heart is measured with a bubble flow meter. The product of the arteriovenous ($A-V$) oxygen-concentration difference and the flow rate gives the oxygen consumption of the heart.

Determination of the oxygen concentration of the effluent from the heart depends on the measurement of the current flowing between a platinum electrode maintained at a suitable negative potential, and a standard non-polarizable anode. The current is produced by the reduction of oxygen molecules to water at the platinum surface, and the magnitude of the current depends on the area of the exposed platinum, the concentration of oxygen in solution, and the prevailing hydrodynamic conditions which determine the rate of diffusion and convection of oxygen molecules to the platinum surface. The chief disadvantages of the method are that the current produced by a constant concentration of oxygen tends to change with time, and that the oxygen current is influenced by the immediate past history of the electrode.

* Present address: Department of Biochemistry, University of Edinburgh.

The experience of many investigators (reviewed by Connelly, 1957) has shown that the most reproducible results are obtained by careful control of the hydrodynamic conditions in the electrode chamber, either by preventing direct access of the exposed platinum to the solution by allowing the oxygen molecules to diffuse through a thin membrane covering the platinum surface, or by allowing the electrode to recover between short periods of polarization (intermittent polarization). As the flow rate of fluid passing through the electrode chamber does not remain constant, a stationary membrane electrode cannot be used for our experiments. Consequently a rotating platinum electrode of the exposed type, similar to the gold electrode used by Longmuir (1954), is used, together with the intermittent-polarization technique. This method has proved reliable, and is suitable for any system in which blood-free solutions flow through an electrode chamber.

*Perfusion method*

The perfusion fluid is a modified Krebs-bicarbonate medium containing half the calcium concentration recommended by Umbreit, Burris & Stauffer (1949). The perfusion apparatus was derived from that used by Bleehen & Fisher (1954), modified by introducing a more efficient method of continuous filtration of the perfusion medium than the Whatman Soxhlet thimbles originally used. In the modified apparatus (Fig. 1) fluid passes into the heart under a hydrostatic pressure of 54 cm $H_2O$, from a small water-jacketed chamber ($R_2$), and is returned to a chamber ($R_1$) at the same level as the first by a gas lift ($G_1$) through which 5% $CO_2$ in oxygen passes. Fluid then passes under a hydrostatic pressure of about 100 cm $H_2O$ to a large sintered-glass filter ($F$) of porosity 4, and the filtered fluid is returned to the chamber ($R_2$) above the heart by a second gas lift ($G_2$). This chamber is sealed and the level of the fluid is kept constant by a siphon ($S$) leading into the chamber ($R_1$) which is open to the atmosphere. A Soxhlet thimble is retained in reservoir $R_1$, as this delays the blocking of the sintered-glass filter. Blocked filters are washed with concentrated sulphuric acid containing a small amount of potassium chlorate and sodium nitrate.

This basic perfusion apparatus was modified for the measurement of the oxygen uptake of the heart by the introduction of two calibration circuits ($C_1$ and $C_2$), and three glass taps ($T_1$, $T_2$ and $T_3$) which allowed fluid from each of the calibration circuits or the heart circuit either to flow straight through, or to pass independently into a water-jacketed electrode chamber ($E$), and then to the appropriate gas lift. A diagram of one of these glass taps in the two positions is shown in Fig. 2, and Fig. 3 shows the three taps connected together and to the electrode chamber. Each calibration circuit is a simplified version of the heart circuit, with the second upper reservoir and the sintered-glass filter omitted. Fluid, the flow of which is restricted by a screw clip, passes from a water-jacketed upper chamber through the glass tap and is returned to the chamber by a gas lift, through which passes a gas mixture containing 5% $CO_2$ and a known percentage of oxygen.

The electrode chamber is sealed at one end with a rubber bung 2 cm in diameter and 0·8 cm. thick, through which passes the platinum electrode. The bung rests against a collar in the glass of the chamber and acts as the fulcrum for the rotating platinum electrode. The walls of the electrode chamber flare out slightly from the collar to allow for the swept volume of the rotated electrode, and a glass tube sealed into the far end of the chamber serves as the fluid inlet. The fluid outlet is near the collar. This design of the electrode chamber reduces the fluid dead space to a minimum.

The electrode consists of a platinum wire sealed into a glass tube 0·6 cm in diameter and 17 cm long. The centre of the bung through which the electrode passes into the chamber is 5 cm from the platinum tip. A steel sleeve is fitted over the far end of the electrode tube and seated in a self-aligning ball-race, which is set eccentrically 0·7 cm. from the centre of a circular brass disk mounted on the motor shaft. The diameter of the circle described by the platinum tip is 0·3 cm. The water-jacketed electrode chamber and motor are mounted rigidly on a wooden board fixed to the bench, making a vibration-free rotating electrode assembly.

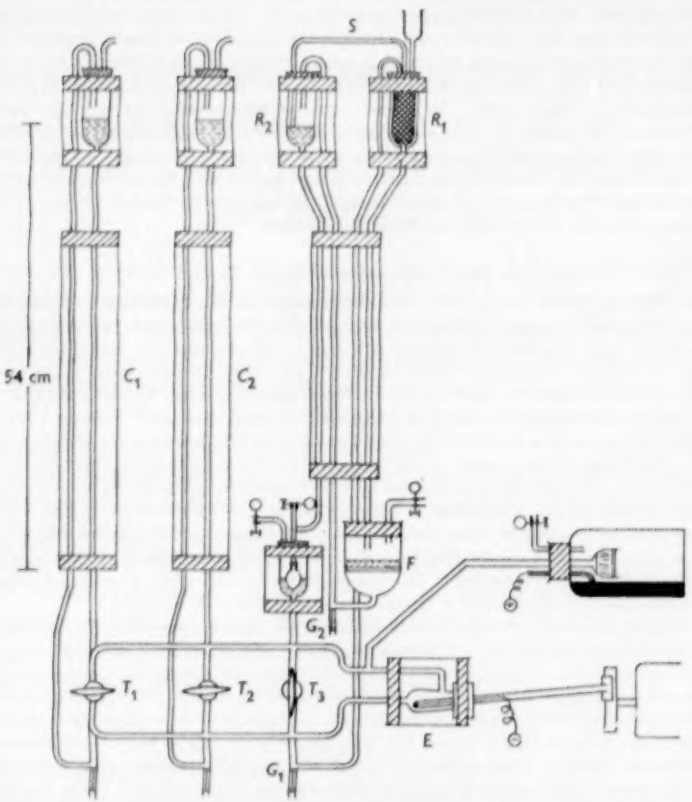



Fig. 1. Arrangement of apparatus for the determination of the oxygen uptake of the perfused rat heart. $C_1$, $C_2$, calibration circuits; *E*, electrode chamber; *F*, filter; $G_1$, $G_2$, gas lifts; $R_1$, $R_2$, water-jacketed chambers; *S*, siphon; $T_1$, $T_2$, $T_3$, glass taps. For further details see text.

*The accuracy and reproducibility of the oxygen electrode*

The chief factors which were found to determine the rate of drift and the reproducibility of the oxygen current were:

*a*, the preparation and care of the electrodes;
*b*, constancy of rotation of the platinum electrode;
*c*, use of an intermittent polarization technique; and
*d*, presence of calcium ions in the solution.

*Preparation and care of the electrodes.* The biggest single factor influencing the stability and reproducibility of the oxygen current undoubtedly resides in the platinum electrode. With the exposed type of platinum electrode used two factors are particularly important. One factor is that careful annealing of the platinum–glass seal prevents the formation of

small cracks in the glass around the seal, visible only under the microscope. The second is freedom from scratches of the platinum surface. The most satisfactory electrodes are made by sealing a short length of 27 s.w.g. Pt wire, which has the end fused into a sphere, into a hard-glass tube of 0·6 cm external diameter, so that just the hemispherical tip is exposed. A length of silver wire is fused to the other end of the platinum wire, and passes out of the glass tube through a small hole 8 cm from the tip. Satisfactory electrodes, not needing the services of a skilled glass-blower, were also made by sealing the platinum–silver wire into

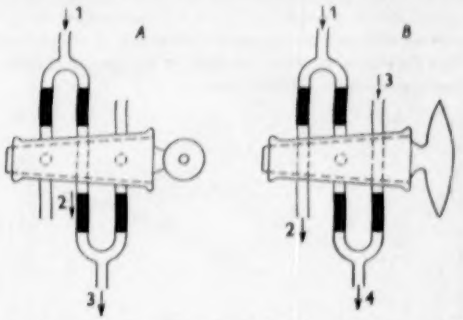



Fig. 2. Special taps for diversion of perfusate to the measuring cell. In *A* perfusate from a calibration circuit or from the heart passes directly to the gas-lift returning it to its reservoir. In *B* it passes to the measuring cell (arrow at bottom left), returns to the tap (arrow at top right) and thence to the gas lift.

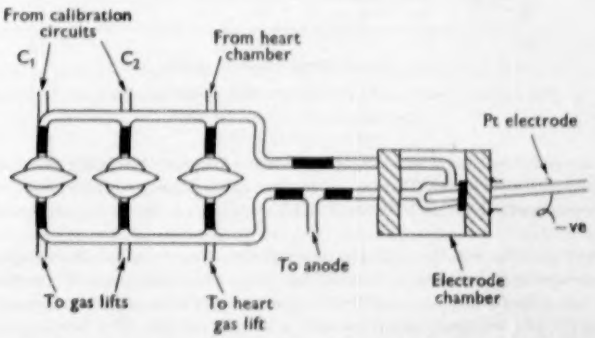



Fig. 3. The connexions of the special taps with the remainder of the apparatus: cf. Fig. 1.

Marco resin 8B26C (Darbre, 1957), using as a mould a glass tube of internal diameter 0·6 cm, coated on the inside with oleic acid as a releasing agent. When not in use electrodes are stored in 10 N nitric acid. Small scratches tend to appear on the platinum surface with use, and these are removed by polishing the surface with jeweller's rouge and then with a fine Perspex polish (No. 3 Perspex polish; I.C.I.).

The type of anode is not critical, provided that it is large enough to eliminate any possibility of anode polarization (Longmuir, 1957), and does not allow diffusion of mercury on to the platinum electrode (Giguère & Lauzier, 1945*a*, *b*). The anode used is a mercury pool of

about 100 cm² surface area, contained in a 500 g bottle resting on its side and filled with saturated KCl solution. A 4 cm coarse sintered-glass filter, cut down to the sintered-glass plate, with the stem partly filled with a 1 cm-thick layer of 4 % agar gel saturated with KCl, serves as a bridge. The bottle is tightly stoppered by a rubber bung, through which projects the stem of the filter, and electrical contact with the mercury is made by a platinum wire sealed into a glass tube, which also passes through the bung.

*Constancy of rotation of the platinum electrode.* Experiments in which the platinum electrode was rotated by a Velodyne motor (Dickinson, 1950) showed that when the fluid was saturated with the gas mixture containing 5 % of $CO_2$ and either 20 % $O_2$ or 95 % $O_2$, the oxygen current increased with increasing speed of rotation of the electrode up to 5000 rev/min (see Fig. 4). This finding is contrary to that of Longmuir (1954), who observed the beginning of a plateau region above 1000 rev/min.

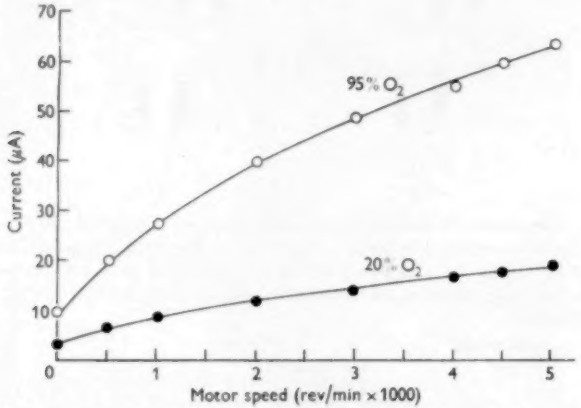



Fig. 4. The relation between speed of rotation of the intermittently polarized electrode and oxygen current.

To ensure a constant speed of rotation, a Fracmo 1/10 h.p. synchronous motor was used to rotate the electrode at 3000 rev/min. At this speed, changes in the flow rate of fluid entering the electrode chamber between 3 and 10 ml./min produce very small changes in the oxygen current.

*Intermittent polarization.* The oxygen current was found to fall in an approximately exponential manner from the time of switching on the polarizing potential, and in some case an hour of pre-polarization at −1·0 V (Longmuir, 1954) was needed before steady values were obtained. The necessary pre-polarization time is reduced to a few minutes, and the stability of the oxygen current is improved, by reducing the average current density at the electrode surface by applying the intermittent polarization technique of Carlson, Brink & Bronk (1950). A 1-rev/min synchronous motor is used to drive a brass cam, which closes two separate micro-switches each for 10 sec in every minute. One micro-switch controls the polarizing potential to the platinum electrode, while the other operates the chart motor of a Cambridge pen-recording polarograph. During the 10 sec period of polarization the pen reaches its maximum excursion, and a series of vertical lines are recorded on the chart at 1 min intervals. The height of the maximum excursion of the recording pen is a linear function of the percentage of oxygen in the equilibrating gas, as is shown in Fig. 5.

*Effect of calcium ions in the perfusion medium.* Before the intermittent-polarization technique was introduced it was observed that the rate of drift of the oxygen current was

considerably greater when the fluid was Krebs-bicarbonate medium than when it was isotonic mixture containing sodium chloride and the same concentration of sodium bicarbonate as the Krebs medium. The difference between the two fluids was found to depend on the presence of calcium in the medium, and it appeared that prolonged exposure of the platinum electrode to solutions containing calcium, and equilibrated with gas mixtures rich in oxygen, leads to the deposition of a film on the platinum surface reducing its effective area. Reduction of the current density at the platinum surface by the intermittent-polarization technique greatly reduces the fall of sensitivity of the electrode with time which this deposition causes.

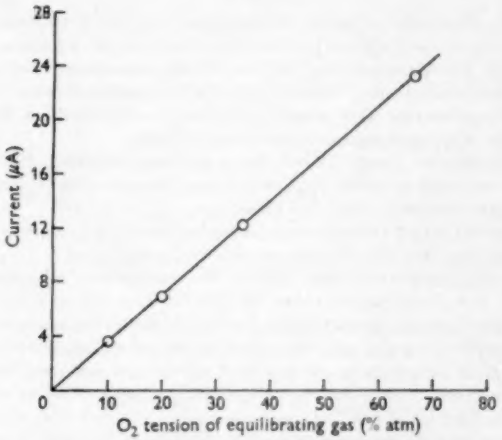



Fig. 5. A typical calibration curve of an electrode rotated at 3000 rev/min, and polarized for 10 sec/min at −0·6 V.

*Current voltage curves*

In common with the other workers using rotating electrodes (Giguère & Lauzier, 1945*b*; Longmuir, 1954) we found no well defined current–voltage plateaux at the speeds of rotation. With a stationary electrode good plateau regions from −0·4 to −1·0 V were obtained, but with the increasing speeds of rotation of the platinum electrode the length of the plateau region decreased, until at 3000 rev/min only a short region of inflexion between 0·6 and −0·8 V remained. However, since the speed of rotation of the electrode is kept constant by the synchronous motor this is not important, and a polarizing potential of −0·6 V is used.

*Application*

Before beginning a perfusion the two calibration circuits and the heart circuit, each containing Krebs-bicarbonate medium, are equilibrated with 5 % $CO_2$ in air, and the flow rates of the calibration circuits are adjusted until the same oxygen current is recorded on each of the three circuits, with the flow rate through the heart circuit set at 6 ml./min. The heart circuit is then equilibrated with 95 % $O_2$ and 5 % $CO_2$, and the two calibration circuits are equilibrated with gas mixtures containing 5 % $CO_2$, and different oxygen contents (usually 20 and 35 %). If the oxygen tension of the effluent from the heart is expected to be greater than 35 % saturation the heart circuit is equilibrated with 5 % $CO_2$ and 70 % $O_2$, so that the oxygen tension of the effluent is within the calibration range. The platinum electrode is calibrated immediately before the start of perfusion, and normally 15 min later and every

subsequent 30 min. The oxygen tension of the effluent is estimated to the nearest $\frac{1}{2}$% of an atmosphere. The flow rate through the perfused heart is measured at 5 min intervals by timing the fall of a gas bubble between two marks on the glass tube conducting fluid into the heart, and the bubble is removed from the circuit by a bubble trap in the cannula. The oxygen uptake of the heart (in $\mu$l./min) is calculated at 5 min intervals, from the product of the arteriovenous oxygen tension difference, the appropriate value of the Bunsen solubility coefficient for oxygen (Dixon, 1951) and the flow rate. The oxygen uptake is converted to $Q_{O_2}$ values ($\mu$l. $O_2$/mg dry wt./hr) after determination of the dry weight of the heart.

*Procedure*

*Animals.* Male albino rats of Wistar stock weighing 200–260 g were used. The rats were bred in the Department and allowed unrestricted access to food and water.

*Perfusion fluids.* The basic perfusion fluid was Krebs–bicarbonate medium (KBM) made up according to the instructions of Umbreit *et al.* (1949), except that the calcium content was halved. Different substrates were added as required to this medium, which was always equilibrated with oxygen gas mixtures containing 5% $CO_2$.

*Insulin.* Crystalline ox insulin (British Drug Houses) containing 23 i.u./mg was used. A stock solution was made up containing 500 m-u./ml., and stored for periods up to 3 months at $-18°$ C as 2 ml. samples in small test tubes.

*Heparin.* Heparin (British Drug Houses) containing 100,000 u./g was used. The dose given was 1 ml. of a solution in NaCl 0·9 g/100 ml., containing 400 u./ml.

*Preparation of the heart for perfusion.* The rat was anaesthetized with ether, and 1 ml. of heparin solution was slowly injected into the femoral vein. After 2 min the thorax was opened and the heart quickly excised and placed in cold KBM. The aorta and branches were cleaned from connective tissue, and the aorta tied on to a glass cannula through which flowed KBM at room temperature. At this stage the heart commenced beating, and blood was quickly washed out of the coronary circulation. The heart, complete with cannula and rubber bung, was then fitted into the heart chamber of the perfusion apparatus, and perfusion commenced, using a pressure head of 54 cm $H_2O$, and a fluid volume of 60–70 ml.

*Estimation of glycogen.* Immediately after perfusion, the heart was taken off the cannula, all tissue above the auriculo-ventricular septum was removed, the ventricles were transected longitudinally and both halves were quickly blotted and weighed on a torsion balance. One half was minced in cold KOH solution, 30 g/100 ml., and the glycogen was separated and hydrolysed by the method of Good, Kramer & Somogyi (1933). The other half was dried overnight in the oven. The glycogen hydrolysate was neutralized with NaOH, 40 g/100 ml., and diluted to 50 ml. The glucose was estimated in this solution by the method of Nelson (1944).

## RESULTS

### *The time course of the oxygen uptake*

Figure 6 shows the change in $Q_{O_2}$ ($\mu$l. $O_2$/mg dry wt./hr) with time of a heart perfused with nutrient-free Krebs-bicarbonate medium (KBM) at 38·5° C and the accompanying changes in the coronary flow and effluent oxygen tension. There is a fall in oxygen consumption over the first 15 min of perfusion and, in parallel with this, a fall in the coronary flow. There is then a long period of steady oxygen consumption during which the coronary flow remains constant. Finally, there is a fall in oxygen consumption. During this time the force of beating of the heart diminishes visibly. This pattern was typical of hearts perfused in the absence of sub-

strate, but the time of onset of the eventual fall in the oxygen uptake varied considerably from heart to heart over the range of 40–90 min from the beginning of perfusion.

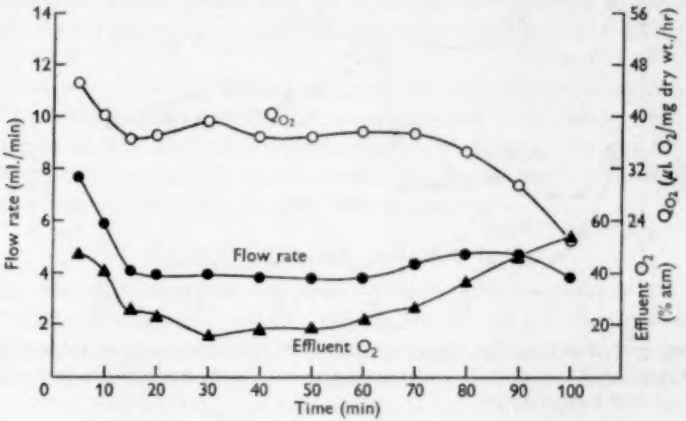



Fig. 6. Measurements at 5 min intervals of oxygen uptake ($Q_{O_2}$ ○), effluent oxygen tension (% atm ▲) and coronary flow rate (ml./min ●).

*Adequacy of oxygen supply*

The fact that there was regularly a measurable oxygen concentration in the coronary effluent (see Fig. 6) is some indication of adequacy of the oxygen supply. But it might be that the heart cannot extract oxygen efficiently below a certain oxygen tension in the fluid supplying it. Experiments of two kinds were made to test this point. In the first set erythritol tetranitrate (final concentration 10 mg/60 ml.) was added after 80 min to a heart perfused at 38·5° C with medium containing 8·3 mM glucose. The erythritol tetranitrate produced a considerable increase in coronary flow, but no systematic increase in the oxygen consumption (see Fig. 7). The effluent oxygen tension also increased considerably, since oxygen supply was greatly increased as the result of the larger coronary flow. In the second set of experiments 2,4-dinitrophenol (DNP) was added to the perfusate 30 min after the start of perfusion, to give a final concentration of $10^{-5}$ M. As Fig. 8 shows, it produces a marked rise in the oxygen consumption and a fall in the effluent oxygen tension, without appreciably affecting the coronary flow. The first of these experiments shows that the heart does not extract more oxygen when the supply is increased by erythritol tetranitrate; the second shows that it can extract more from the normal coronary flow when its metabolism is stimulated by DNP. Thus the normal effluent oxygen tension is not so low as to limit oxygen uptake.

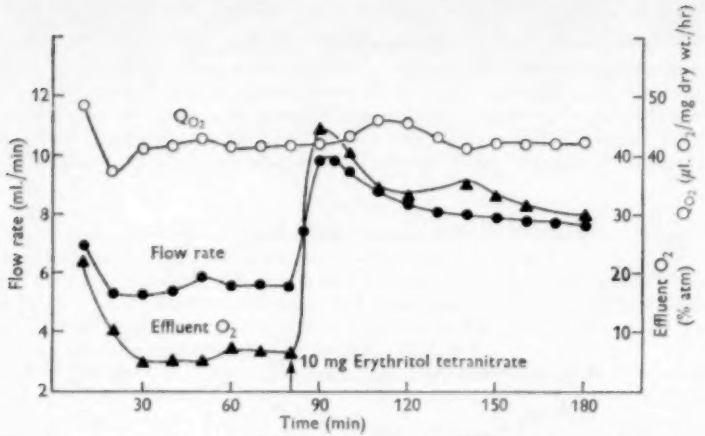



**Fig. 7.** The effect of erythritol tetranitrate (10 mg in 60 ml.) on $Q_{O_2}$ (○), coronary flow rate (ml./min ●) and on effluent oxygen tension (% atm ▲). The perfusate contained 8·3 mM glucose.

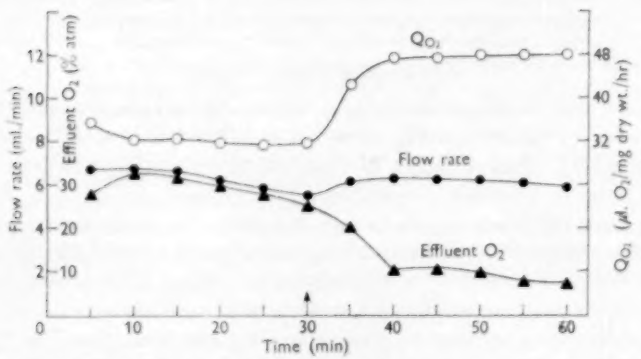



**Fig. 8.** The effect of 2,4-dinitrophenol (DNP, $10^{-4}$ M, at arrow) on the $Q_{O_2}$ (○), coronary flow rate (ml./min ●) and effluent oxygen tension (% atm ▲) of a heart perfused with 8·3 mM glucose.

*Completeness of equilibration of perfusate with gas mixture*

The two quantities measured are the oxygen concentrations in the coronary effluent and the coronary flow. The A–V oxygen concentration difference is calculated on the assumption that the perfusate returned to the heart reservoir is in equilibrium with the gas mixture used in the gas lifts. If there is an error in this assumption, i.e. if the perfusate is less than saturated, increase in coronary flow will produce a fall in the calculated

oxygen consumption. Also, if equilibration is imperfect, alteration of the oxygen content of the equilibrating gas mixture should change the calculated oxygen uptake. The experiments with erythritol tetranitrate show that the first of these effects does not occur, and several trials with changes of the oxygen tension of the gas mixture have failed to detect the second effect.

A third way of testing this point is to immerse a small sintered-glass gas-bubbler in the perfusate reservoir supplying the heart, and to examine the effect on the apparent oxygen uptake of passing the gas mixture through this as well as through the gas-lifts. No diminution in calculated oxygen uptake was observed when this was done.

*Effect of temperature on oxygen uptake*

The range of temperature studied was small. In view of the initial drop in oxygen consumption, and the decline in the oxygen consumption of some hearts perfused with nutrient-free medium after 40 min, the oxygen uptake in the period 15–40 min is taken as characteristic of the steady state of the heart. The mean $Q_{O_2}$ of eight hearts at 34° C was $32{\cdot}8 \pm 0{\cdot}9$, and the mean $Q_{O_2}$ of thirteen hearts at 38·5° C was $43{\cdot}9 \pm 0{\cdot}9$. In general it is undesirable to perfuse at the higher temperature, since, on occasion, the coronary flow may be too low to give a comfortable margin of oxygen supply. The increase in oxygen consumption with increase in temperature corresponds to a $Q_{10}$ of 2·31, which gives an estimated $Q_{O_2}$ of 38·8 at 37° C.

*The endogenous substrate for oxidation*

Experiments were performed in which hearts were perfused at 37° C for varying periods up to 60 min with nutrient-free KBM in the presence and absence of 2 m-u./ml. insulin. The glycogen contents of these hearts were then determined. The results, shown in Table 1, indicate that the glycogen content falls steadily throughout the first hour of perfusion, and that insulin has no effect on the course of this fall.

In other experiments hearts were made anoxic by perfusing for different periods of time with the perfusion fluid equilibrated with 5% $CO_2$ in nitrogen before transferring the hearts to fully oxygenated perfusate, and continuing perfusion for a total time of 15 min. The results of glycogen determinations on these hearts are given in Table 2. In one series the hearts were anoxic for a standard time of 10 min, by which time they had all ceased to beat. On transference to the oxygenated medium beating recommenced within 1 min, and at the end of 5 min all the hearts were beating forcibly. In another series the hearts were kept anoxic until they ceased to beat, and were then immediately transferred to the oxygenated medium. Table 2 shows that the apparent amount of glycogen content of the hearts

was not dependent on the period of anoxia, but that in each case the glycogen content was very low, and at much the same level as the glycogen content of the hearts perfused aerobically for 60 min.

The apparent residual glycogen after 15 min anoxic or 60 min aerobic perfusion is not in fact glycogen. Determination of the apparent glucose in final hydrolysates by the glucose oxidase method (Huggett & Nixon, 1957)

TABLE 1. Time course of the glycogen disappearance from rat hearts perfused with nutrient-free medium at 37° C

| Time of perfusion (min) | Apparent glycogen content as glucose (mg/g dry wt.) | |
|---|---|---|
| | Nutrient-free medium | Nutrient-free medium containing insulin 2 m-u./ml. |
| 0 | 16·3 ± 1·1 (16) | 16·3 ± 1·1 (16) |
| 15 | 13·1 ± 1·8 (8) | 12·4 ± 1·7 (12) |
| 30 | 10·5 ± 1·3 (4) | 8·1 ± 1·0 (6) |
| 60 | 3·6 ± 0·9 (4) | 4·1 ± 0·9 (4) |

Standard errors of means are shown; number of experiments given in parentheses.

TABLE 2. Glycogen content of the rat heart after anoxia. The perfusion medium was equilibrated with 95 % $N_2$ + 5 % $CO_2$ during anoxia and the heart was transferred to a second perfusion apparatus equilibrated with 95 % $O_2$ + 5 % $CO_2$ and perfused for a total period (anaerobic + aerobic) of 15 min

| Apparent glycogen content as glucose (mg/g dry wt.) after 15 min perfusion | Duration of anoxia (min) |
|---|---|
| 2·1 | 10 |
| 3·1 | 10 |
| 2·7 | 10 |
| 2·4 | 10 |
| Mean = 2·3 ± 0·4 | |
| 2·0 | 4 |
| 3·5 | 8 |
| 3·5 | 7 |
| 3·5 | 7 |
| Mean = 3·2 ± 0·4 | |

Standard errors of the mean are shown.

has shown that there is consistently less glucose present than is indicated by the Nelson (1944) method. In a large series it has been shown that the mean difference is 2·73 + 0·21 (23) mg/g dry wt., and this difference is independent of total apparent glycogen in the heart. Most, if not all, of the apparent residual glycogen found in the experiments reported in Table 2 is therefore not glycogen.

*Effect of anoxia on survival*

Figure 9 shows the effect on the $Q_{O_2}$, flow rate, and effluent oxygen tension of changing the oxygen tension of the gas equilibrating the perfusion medium from 70% saturation to 20% saturation and back again. The $Q_{O_2}$ was reduced to about 40% of its original value during the 20 min period of partial anoxia, but recovery was complete. The oxygen tension of the effluent fell from 20% saturation to zero, indicating a complete

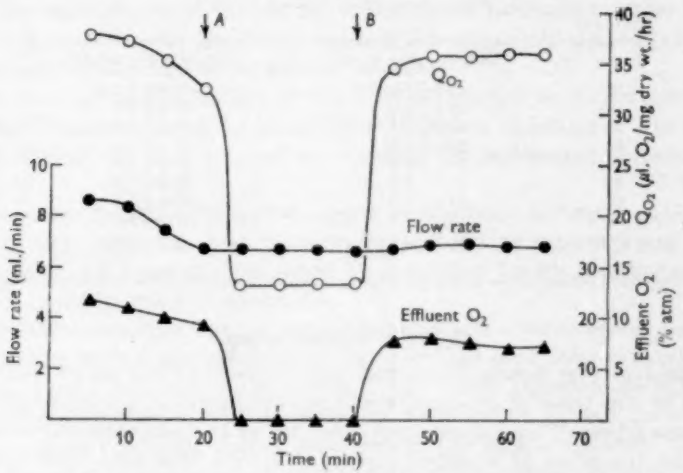



Fig. 9. The effect of altering the oxygen content of the equilibrating gas on $Q_{O_2}$ (○), coronary flow rate (ml./min ●), and effluent oxygen tension (▲). At the beginning and end of the experiment the gas is 5% $CO_2$ in oxygen. Between arrows *A* and *B* it is 5% $CO_2$ in air.

extraction of all the available oxygen, but the flow rate did not increase to compensate for the lower oxygen availability. In this experiment the perfusion medium contained 8·3 mM glucose, and the temperature was 37° C, but similar results were obtained with other perfusion media, and when the period of partial anoxia occurred at a later stage during perfusion.

In other experiments hearts were perfused with nutrient-free KBM equilibrated with 5% $CO_2$ in nitrogen until they stopped beating, and were then transferred to the apparatus for determination of oxygen consumption and perfused for a further 60 min with the nutrient-free KBM equilibrated with 5% $CO_2$ in oxygen. The time course of the oxygen consumption of these hearts is compared with that of hearts perfused from the beginning with oxygenated medium in Table 3.

All the hearts commenced to beat vigorously within 1 min after instituting aerobic perfusion but the mean $Q_{O_2}$ during the period 0–60 min is only about 70% of that of the controls over the corresponding period, although oxygen consumption in the two series of hearts follows similar time courses. The mean rate of oxygen uptake in both series of the experiments is fairly steady from 10 to 50 min of perfusion, but shows a marked fall after 50 min.

TABLE 3. Effect of anoxia on the oxygen consumption of the isolated rat heart perfused with nutrient-free medium at 37° C

| Time of perfusion (min) | $Q_{O_2}$ of normal hearts (ml./mg dry wt./hr) | $Q_O$ after anoxia (ml./mg dry wt./hr) |
|---|---|---|
| 5 | 45·2 ± 1·8 (22) | 29·8 ± 1·7 (4) |
| 10 | 42·7 ± 1·6 | 27·6 ± 1·6 |
| 15 | 40·0 ± 1·5 | 26·9 ± 1·7 |
| 20 | 39·4 ± 1·3 | 27·3 ± 1·5 |
| 30 | 38·5 ± 1·3 | 29·6 ± 1·5 |
| 40 | 37·5 ± 1·4 | 29·2 ± 1·3 |
| 50 | 36·8 ± 1·6 | 28·4 ± 1·2 |
| 60 | 34·9 ± 1·8 | 25·2 ± 1·0 |
| Mean 0–60 min | 39·4 ± 1·5 | 28·0 ± 1·4 |

Standard errors of the mean are shown; number of experiments in parentheses.

### *Effect of repeated anoxia*

The fact that hearts which had been exposed to anoxia for sufficient time to free them of glycogen would recover apparently completely, and resume a beat of normal rate and force, led us to make some experiments to test the effect of repeated periods of anoxia. The first heart studied ceased to beat after 6 min of anoxia, recovered rapidly when perfused with oxygenated medium, ceased to beat 5 min after switching to anoxic perfusate, recovered again within 5 min and continued to beat strongly for some time. Two further hearts treated similarly ceased to beat 1 min after the beginning of the second period of anoxia, recovered within 1–2 min after reinstituting aerobic perfusion 20–30 min later, but became feeble in approximately 20 min. Each of these hearts was exposed to nitrogen-equilibrated perfusate during the first period of anoxia for long enough to deplete it effectively of glycogen, so that it is difficult to see what source of energy was available to the last two hearts during their second prolonged period of anoxia. It seems probable that, as long as the heart is not performing external work, it can maintain the organization on which a propagated beat depends for some time after it is deprived of a source of energy, even when it is maintained at normal body temperature.

## DISCUSSION

The most important finding in this work is that when an isolated heart of the rat, an organ which might be expected to have a high metabolic rate, is perfused with a saline medium of low oxygen capacity, it is possible to provide a margin of oxygen supply over requirement, provided that the perfusion temperature is not too high. (The upper limit of temperature of adequate oxygenation is probably 38·5–39° C.)

The tests described of the adequacy of oxygenation of the medium show that the equilibration of the gas mixture with the perfusate must be very nearly complete in our conditions and that, in consequence, our estimates of oxygen uptake may be considered reliable.

The level of oxygen uptake of 37° C, 39 $\mu$l./mg dry wt./hr, is much in excess of figures quoted for heart slices (Pearson, Hastings & Bunting, 1949), though the hearts in our experiments are performing no external work.

The reason for the initial fall in oxygen consumption has not been determined. It is suggestive that there are parallel falls in coronary flow and heart rate. All these changes could be accounted for by destruction or elution from the heart of adrenaline.

The maintenance of the oxygen consumption for a considerable period of perfusion with a simple saline perfusate implies that there is available a large supply of endogenous nutrient. The mean glycogen content of hearts before perfusion is 13–14 mg/g dry wt. (data of Table 1 corrected for non-glucose reducing matter measured by the Nelson method). Complete oxidation of this would supply the heart with substrate for 14–19 min at the observed rates of oxygen consumption. As the respiration rarely falls off seriously within an hour, the majority of the substrate must be something other than glycogen and probably other than carbohydrate.

Measurements of glycogen after different times of perfusion show that it falls off linearly with time, disappearing after the first hour. During this time its metabolism would account for 20–30% of the oxygen consumed. It is of interest, that when hearts are made anoxic initially, the whole of the glycogen disappears and the oxygen uptake when the hearts are subsequently perfused aerobically is in the region of 70% of that of hearts perfused aerobically throughout the experiment. It looks as though two independent kinds of oxidative metabolism were going on side by side.

A point of some interest arises when one compares the oxygen uptake of these hearts with other estimates of heart oxygen uptake in the literature. No other figures are available for the rat heart, but values are available for blood-perfused cat and dog hearts. Extrapolating the results of Evans & Matsuoka (1915) to conditions of zero external work, one gets a $Q_{O_2}$ of 14

for the dog heart. The data of Laurent, Bolene–Williams, Williams & Katz (1956) for the dog heart beating at 100/min and doing no external work yield a $Q_{O_2}$ of 13. A Langendorff perfusion of the dog heart used by van Citters, Ruth & Reissmann (1957) gives a $Q_{O_2}$ of 9 for the same conditions. Lorber (1953) found a $Q_{O_2}$ of 15 for the perfused cat heart in diastolic arrest. If we take it that the metabolic rate per unit mass of similar tissues is proportional to the third or fourth root of body weight (Brody, 1945), these data are comparable to the present figures for rat hearts. This suggests that neither the nutrients supplied by blood, such as lactate, nor such other major constituents as are missing from our saline perfusate, such as proteins and lipids, have any marked effect on the oxygen uptake.

It appears possible therefore that perfusion of larger—and more slowly metabolizing—hearts with oxygenated saline solution might supply them adequately for considerable periods of time, so long as they were not performing external work.

## SUMMARY

1. A method is described for the determination of the oxygen consumption of the saline-perfused rat heart.

2. The oxygen consumption, coronary flow rate and heart rate are found to fall during the first 20 min of perfusion. Thereafter they stay steady for approximately 40 min.

3. During this period the glycogen content of the heart declines linearly.

4. During the period of steady oxygen consumption the $Q_{O_2}$ at 37° C is 39 and the $Q_{10}$ of oxygen uptake is 2·3.

5. Perfusion for 4–10 min with saline perfusate equilibrated with 5% $CO_2$ in $N_2$ results in complete disappearance of glycogen from the heart.

6. Hearts so treated show normal rate and force of beat within a few minutes of reinstitution of aerobic perfusion.

7. In a few instances it was shown that such hearts can withstand a second period of anaerobic perfusion, lasting as long as 20–30 min.

8. It is concluded (*a*) that the heart has a large reserve of oxidizable material other than glycogen and (*b*) that the cardiac organization on which the propagated beat is dependent is not readily disintegrated when metabolic energy supplies are withdrawn.

## REFERENCES

Bleehen, N. M. & Fisher, R. B. (1954). The action of insulin in the isolated rat heart. *J. Physiol.* **123**, 260–276.

Brody, S. (1945). *Bioenergetics and growth.* New York: Reinhold.

Carlson, F. D., Brink, F. & Bronk, D. W. (1950). A continuous flow respirometer utilizing the oxygen cathode. *Rev. sci. Instrum.* **21**, 923–932.

Connelly, C. M. (1957). Methods for measuring tissue oxygen tension; theory and evaluation: the oxygen electrode. *Fed. Proc.* **16**, 681–684.

DARBRE, A. (1957). A plastic homogenizer. *J. Physiol.* **136**, 2*P*.

DAVIES, P. W. & BRINK, F. (1942). Microelectrodes for measuring local oxygen tension in animal tissues. *Rev. sci. Instrum.* **13**, 524–533.

DICKINSON, C. J. (1950). *Electrophysiological technique*, p. 105. London: Morgan.

DIXON, M. (1951). *Manometric Methods*, 3rd ed. Cambridge University Press.

EVANS, C. L. & MATSUOKA, Y. (1915). The effect of various mechanical conditions on the gaseous metabolism and efficiency of the mammalian heart. *J. Physiol.* **49**, 378–405.

GIGUÈRE, P. A. & LAUZIER, L. (1945*a*). Le dosage polarographique de l'oxygène dissous dans l'eau de mer. *Canad. J. Res.* B, **23**, 76–83.

GIGUÈRE, P. A. & LAUZIER, L. (1945*b*). Analyse polarometrique de l'oxygène dissous avec une micro-electrode de platine. *Canad. J. Res.* B, **23**, 223–233.

GOOD, C. H., KRAMER, H. & SOMOGYI, M. (1933). The determination of glycogen. *J. biol. Chem.* **100**, 485–491.

HUGGETT, A. St G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase and *o*-dianisidine in determination of blood and urinary glucose. *Lancet*, **273**, 368–370.

LAITINEN, H. A. & KOLTHOFF, I. M. (1941*a*). Voltammetry with stationary microelectrodes of platinum wire. *J. phys. Chem.* **45**, 1061–1079.

LAITINEN, H. A. & KOLTHOFF, I. M. (1941*b*). Voltammetric determination and amperometric titrations with a rotating microelectrode of platinum wire. *J. phys. Chem.* **45**, 1079–1093.

LAURENT, D., BOLENE-WILLIAMS, C., WILLIAMS, F. L. & KATZ, L. N. (1956). Effects of heart rate on coronary flow and cardiac oxygen consumption. *Amer. J. Physiol.* **185**, 355–364.

LONGMUIR, I. S. (1954). Respiration rate of bacteria as a function of oxygen concentration. *Biochem. J.* **57**, 81–87.

LONGMUIR, I. S. (1957). Respiration rate of rat-liver cells at low oxygen concentrations. *Biochem. J.* **65**, 378–382.

LORBER, V. (1953). Energy metabolism of the completely isolated mammalian heart in failure. *Circ. Res.* **1**, 298–311.

NELSON, N. J. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375–380.

PEARSON, O. H., HASTINGS, A. B. & BUNTING, H. (1949). Metabolism of cardiac muscle; utilization of $^{14}C$ labelled pyruvate and acetate by heart slices. *Amer. J. Physiol.* **158**, 251–260.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1949). *Manometric techniques and tissue metabolism.* Minneapolis: Burgess.

VAN CITTERS, R. L., RUTH, W. E. & REISSMANN, K. R. (1957). Effect of heart rate on oxygen consumption of isolated dog heart performing no external work. *Amer. J. Physiol.* **191**, 443–445.

*J. Physiol.* (1961), **158**, *pp.* 102–112
*With 2 text-figures*
*Printed in Great Britain*

# THE EFFECTS OF INSULIN, ADRENALINE AND NUTRIENTS ON THE OXYGEN UPTAKE OF THE PERFUSED RAT HEART

By R. B. FISHER* and J. R. WILLIAMSON
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

The isolated perfused rat-heart preparation developed by Bleehen & Fisher (1954) has in principle several advantages over other preparations for the study of the effect of hormones on the metabolism of muscle tissue. It has the advantage over the more classical heart-lung preparation of simplicity, and is at the same time a more physiological preparation than heart slices, diaphragm or skeletal muscle. In the perfused heart preparation the oxygen tension of the extracellular fluid is higher and more uniform than that of incubated tissues, where fluid of high oxygen tension impinges directly only on the two outermost layers of cells, and where it may be necessary for oxygen to diffuse through eight or more layers of cells to reach the centre of the tissue. Creese, Scholes & Whalen (1958) have demonstrated that the resting potentials of the innermost layers of cells in incubated rat diaphragm are very sensitive to the oxygen tension of the bathing fluid, and that adequate oxygenation is difficult to achieve. Since the lowering of the resting potential is an acute phenomenon, it is likely that the metabolism of incubated tissues may on occasion be limited by oxygen availability.

Bleehen & Fisher (1954) have shown that the glucose uptake of the perfused rat heart is greatly increased by insulin, and only part of the extra glucose uptake is deposited as glycogen during the observed experimental period (Fisher & Lindsay, 1956), suggesting that insulin increases the amount of glucose oxidized. The original purpose of this work was to investigate the increase in oxygen uptake that might be expected as the result of the action of insulin.

In fact, there is no such increase in the oxygen uptake, and the oxygen consumption of the heart remains remarkably constant whatever the nature of the substrate provided, which suggests that endogenous and exogenous substrates compete with each other for oxidation and that insulin affects the proportion of glucose metabolized relative to the total metabolism. The oxygen consumption of the heart is, however, markedly

* Present address: Department of Biochemistry, University of Edinburgh.

increased by adrenaline and 2,4-dinitrophenol (DNP), so that it is clear that the absence of a stimulating effect of insulin on oxygen consumption is not due to an inability of the preparation to respond to stimulation.

## METHODS

The perfusion technique and the method of determination of the oxygen uptake of the perfused rat heart have been described (Fisher & Williamson, 1961). Male rats of Wistar stock weighing 220–280 g were used.

*Acetoacetate* was used as the sodium salt, prepared by the method of Krebs & Eggleston (1945), and standardized by the aniline-citrate method of Edson (1935).

*β-Hydroxybutyrate.* The racemic sodium salt (British Drug Houses) was used.

*2,4-Dinitrophenol.* A suitable volume of stock $10^{-3}$ M solution of DNP (British Drug Houses) was added to the perfusate, to make the final concentration approximately $10^{-5}$ M. Spectroscopic analysis of the perfusate showed that the DNP concentration varied from 0·9 to $1{\cdot}2 \times 10^{-5}$ M in different experiments.

*Adrenaline.* A solution of adrenaline (Boots, Ltd; 10 μg/ml.) in saline (0·9 g NaCl/100 ml.) was prepared and stored in the deep freeze in 2 ml. portions. 1 ml. of this adrenaline solution was added to a perfusate volume of approximately 60 ml.

*Insulin.* Crystalline ox insulin (British Drug Houses) was used containing 23 u./mg. A stock solution containing 200 m-u./ml. was made up in 0·9 % NaCl and stored as small samples in the deep freeze for periods up to 3 months.

## RESULTS

### *Effect of glucose and glucose with insulin on the endogenous respiration of the perfused rat heart*

The time course of the oxygen uptake of rat hearts perfused with Krebs-bicarbonate medium (KBM) containing no added substrate, 8·3 mM glucose, and 8·3 mM glucose + insulin 2 m-u./ml., was followed for 60 min at temperatures of 38·5 and 35° C. The results are presented in Table 1. It is seen that the mean $Q_{O_2}$ of hearts at the two temperatures follows similar time courses, irrespective of the nature of the perfusion medium, falling slightly during the first quarter of an hour of perfusion, and then remaining steady for the 60 min perfusion period. When the perfusion medium contained no substrate there was a tendency for the $Q_{O_2}$ to fall towards the end of 60 min. Glucose, or glucose with insulin, has no significant effect on the endogenous respiration of the perfused rat heart, as is seen from the mean $Q_{O_2}$ values over the period from 15 to 60 min. $Q_{O_2}$ is markedly influenced by temperature. By pooling the mean $Q_{O_2}$ values for each of the three perfusates a mean $Q_{O_2}$ of $43{\cdot}2 \pm 0{\cdot}7(38)$ μl. $O_2$/mg dry wt./hr is obtained at 38·5° C, and a mean $Q_{O_2}$ of $32{\cdot}3 \pm 0{\cdot}6(25)$ at 35° C, giving a $Q_{10}$ of 2·3. Using this temperature coefficient, a mean $Q_{O_2}$ of 38 is derived for the respiration of the perfused rat heart at 37° C. Measurement of the oxygen uptake of the rat heart at 38·5° C at 10 min intervals

*J. Physiol.* (1961), **158**, *pp.* 102–112
*With 2 text-figures*
*Printed in Great Britain*

# THE EFFECTS OF INSULIN, ADRENALINE AND NUTRIENTS ON THE OXYGEN UPTAKE OF THE PERFUSED RAT HEART

By R. B. FISHER* and J. R. WILLIAMSON
*From the Department of Biochemistry, University of Oxford*

(*Received* 13 *February* 1961)

The isolated perfused rat-heart preparation developed by Bleehen & Fisher (1954) has in principle several advantages over other preparations for the study of the effect of hormones on the metabolism of muscle tissue. It has the advantage over the more classical heart-lung preparation of simplicity, and is at the same time a more physiological preparation than heart slices, diaphragm or skeletal muscle. In the perfused heart preparation the oxygen tension of the extracellular fluid is higher and more uniform than that of incubated tissues, where fluid of high oxygen tension impinges directly only on the two outermost layers of cells, and where it may be necessary for oxygen to diffuse through eight or more layers of cells to reach the centre of the tissue. Creese, Scholes & Whalen (1958) have demonstrated that the resting potentials of the innermost layers of cells in incubated rat diaphragm are very sensitive to the oxygen tension of the bathing fluid, and that adequate oxygenation is difficult to achieve. Since the lowering of the resting potential is an acute phenomenon, it is likely that the metabolism of incubated tissues may on occasion be limited by oxygen availability.

Bleehen & Fisher (1954) have shown that the glucose uptake of the perfused rat heart is greatly increased by insulin, and only part of the extra glucose uptake is deposited as glycogen during the observed experimental period (Fisher & Lindsay, 1956), suggesting that insulin increases the amount of glucose oxidized. The original purpose of this work was to investigate the increase in oxygen uptake that might be expected as the result of the action of insulin.

In fact, there is no such increase in the oxygen uptake, and the oxygen consumption of the heart remains remarkably constant whatever the nature of the substrate provided, which suggests that endogenous and exogenous substrates compete with each other for oxidation and that insulin affects the proportion of glucose metabolized relative to the total metabolism. The oxygen consumption of the heart is, however, markedly

* Present address: Department of Biochemistry, University of Edinburgh.

increased by adrenaline and 2,4-dinitrophenol (DNP), so that it is clear that the absence of a stimulating effect of insulin on oxygen consumption is not due to an inability of the preparation to respond to stimulation.

## METHODS

The perfusion technique and the method of determination of the oxygen uptake of the perfused rat heart have been described (Fisher & Williamson, 1961). Male rats of Wistar stock weighing 220–280 g were used.

*Acetoacetate* was used as the sodium salt, prepared by the method of Krebs & Eggleston (1945), and standardized by the aniline-citrate method of Edson (1935).

*β-Hydroxybutyrate.* The racemic sodium salt (British Drug Houses) was used.

*2,4-Dinitrophenol.* A suitable volume of stock $10^{-3}$ M solution of DNP (British Drug Houses) was added to the perfusate, to make the final concentration approximately $10^{-5}$ M. Spectroscopic analysis of the perfusate showed that the DNP concentration varied from 0·9 to $1{\cdot}2 \times 10^{-5}$ M in different experiments.

*Adrenaline.* A solution of adrenaline (Boots, Ltd; 10 μg/ml.) in saline (0·9 g NaCl/100 ml.) was prepared and stored in the deep freeze in 2 ml. portions. 1 ml. of this adrenaline solution was added to a perfusate volume of approximately 60 ml.

*Insulin.* Crystalline ox insulin (British Drug Houses) was used containing 23 u./mg. A stock solution containing 200 m-u./ml. was made up in 0·9% NaCl and stored as small samples in the deep freeze for periods up to 3 months.

## RESULTS

### *Effect of glucose and glucose with insulin on the endogenous respiration of the perfused rat heart*

The time course of the oxygen uptake of rat hearts perfused with Krebs-bicarbonate medium (KBM) containing no added substrate, 8·3 mM glucose, and 8·3 mM glucose + insulin 2 m-u./ml., was followed for 60 min at temperatures of 38·5 and 35° C. The results are presented in Table 1. It is seen that the mean $Q_{O_2}$ of hearts at the two temperatures follows similar time courses, irrespective of the nature of the perfusion medium, falling slightly during the first quarter of an hour of perfusion, and then remaining steady for the 60 min perfusion period. When the perfusion medium contained no substrate there was a tendency for the $Q_{O_2}$ to fall towards the end of 60 min. Glucose, or glucose with insulin, has no significant effect on the endogenous respiration of the perfused rat heart, as is seen from the mean $Q_{O_2}$ values over the period from 15 to 60 min. $Q_{O_2}$ is markedly influenced by temperature. By pooling the mean $Q_{O_2}$ values for each of the three perfusates a mean $Q_{O_2}$ of $43{\cdot}2 \pm 0{\cdot}7(38)$ μl. $O_2$/mg dry wt./hr is obtained at 38·5° C, and a mean $Q_{O_2}$ of $32{\cdot}3 \pm 0{\cdot}6(25)$ at 35° C, giving a $Q_{10}$ of 2·3. Using this temperature coefficient, a mean $Q_{O_2}$ of 38 is derived for the respiration of the perfused rat heart at 37° C. Measurement of the oxygen uptake of the rat heart at 38·5° C at 10 min intervals

over 2 hr (Table 2) shows that there is a tendency for the $Q_{O_2}$ to fall in the last half hour.

The stability of oxygen uptake during the middle period of 60 min and its independence of glucose and insulin is well shown in Fig. 1. Although the $Q_{O_2}$ of hearts perfused without added substrate invariably fell before

TABLE 1. Effect of glucose, and of glucose with insulin, on the endogenous $Q_{O_2}$ of the perfused rat heart

| | $Q_{O_2}$ (μl. $O_2$/mg dry wt./hr)* with additions to perfusion medium | | | | | |
|---|---|---|---|---|---|---|
| | None | | Glucose 150 mg/100 ml. | | Glucose 150 mg/100 ml. + Insulin 2 m-u./ml. | |
| Temp (° C) ... | 38·5 | 35 | 38·5 | 35 | 38·5 | 35 |
| No. of expts. ... | 13 | 9 | 14 | 9 | 11 | 7 |
| Time of perfusion (min) | | | | | | |
| 5 | 52·5 ± 2·6 | 36·8 ± 2·5 | 50·0 ± 1·1 | 33·6 ± 1·5 | 51·0 ± 1·8 | 36·7 ± 3·4 |
| 10 | 48·9 ± 2·2 | 34·4 ± 2·2 | 45·9 ± 1·0 | 32·2 ± 1·4 | 47·1 ± 1·2 | 34·1 ± 3·0 |
| 15 | 45·0 ± 2·2 | 34·0 ± 1·9 | 43·4 ± 0·9 | 31·1 ± 1·1 | 45·4 ± 1·4 | 34·4 ± 2·8 |
| 20 | 44·2 ± 1·8 | 33·9 ± 2·1 | 42·5 ± 1·1 | 31·5 ± 1·2 | 43·5 ± 2·0 | 34·3 ± 2·6 |
| 30 | 43·8 ± 1·5 | 32·4 ± 2·4 | 42·6 ± 1·5 | 32·1 ± 1·2 | 42·7 ± 1·7 | 32·6 × 2·1 |
| 40 | 42·6 ± 1·9 | 31·9 ± 2·1 | 42·0 ± 1·7 | 32·5 ± 0·9 | 43·8 ± 1·4 | 32·0 ± 2·0 |
| 50 | 41·1 ± 2·7 | 31·9 ± 1·8 | 42·7 ± 1·5 | 32·2 ± 0·9 | 44·9 ± 1·5 | 31·6 ± 2·1 |
| 60 | 39·5 ± 3·5 | 29·5 ± 1·5 | 42·6 ± 1·5 | 32·0 ± 0·9 | 45·3 ± 1·6 | 31·0 ± 2·3 |
| Mean (15–60 min) | 42·7 ± 0·8 | 32·3 ± 0·8 | 42·6 ± 0·6 | 31·9 ± 0·5 | 44·3 ± 0·7 | 32·6 ± 0·6 |

* Mean ± standard error of the mean.

TABLE 2. Effect of glucose and of glucose with insulin on the endogenous $Q_{O_2}$ of the rat heart over a 2 hr period of perfusion at 38·5° C

| | $Q_{O_2}$ (μl. $O_2$/mg dry wt./hr)† with additions to perfusion medium | | |
|---|---|---|---|
| Time of perfusion (min) | None | Glucose 150 mg/100 ml. | Glucose 150 mg/100 ml + Insulin 2 m-u./ml. |
| 10–30 | 43·4 ± 1·8 | 44·9 ± 1·1 | 45·9 ± 1·4 |
| 40–60 | 41·8 ± 2·5 | 43·4 ± 1·0 | 44·4 ± 1·4 |
| 70–90 | 41·7 ± 3·3 | 43·0 ± 1·3 | 43·3 ± 1·7 |
| 100–120 | 37·2 ± 2·7 | 40·8 ± 1·8 | 38·1 ± 2·0 |

† Mean, standard error of the mean,; 4 expts. in each column.

90 min, the $Q_{O_2}$ of some hearts perfused with glucose remained steady for up to 3 hr, and the fall in the $Q_{O_2}$ of some hearts in the presence of glucose is probably due to faulty perfusion, since it was always accompanied by a fall in the flow rate.

As a check of any possible small effect of insulin on the oxygen uptake of the rat heart perfused with glucose medium, insulin was added after 60 min to three hearts perfused with 8·3 mM glucose, to give a final insulin concentration in the perfusion medium of 2 m-u./ml., and the oxygen consumption was measured at 10 min intervals over a further period of 1 hr

(Table 3). These hearts showed no indication of a fall in the $Q_{O_2}$ after 90 min, and the mean $Q_{O_2}$ over the first hour of perfusion in the absence of insulin was the same as that over the second hour of perfusion in the presence of insulin.

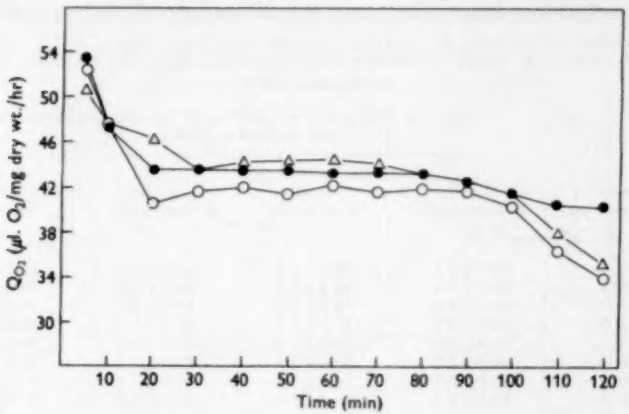



**Fig. 1.** The lack of effect of glucose or of insulin on the oxygen uptake of the heart: 38·5° C. ○ Nutrient-free (4); ● 8·3 mM glucose (4); △ 8·3 mM glucose + 2 m-u. insulin (4). Numbers of experiments in brackets.

**TABLE 3.** Effect on the mean $Q_{O_2}$ of the addition of insulin 2 m-u./ml. at 60 min to three hearts perfused with glucose 150 mg/100 ml. at 38·5° C

| Time of perfusion (min) | $Q_{O_2}$ (µl. $O_2$/mg dry wt./hr) | Time of perfusion (min) | $Q_{O_2}$ (µl. $O_2$/mg dry wt./hr) |
|---|---|---|---|
| 10 | 47·1 ± 1·4 | 70 | 47·4 ± 1·5 |
| 20 | 44·0 ± 2·3 | 80 | 47·0 ± 0·6 |
| 30 | 45·8 ± 3·1 | 90 | 45·5 ± 1·2 |
| 40 | 46·8 ± 2·7 | 100 | 45·7 ± 1·2 |
| 50 | 46·8 ± 2·7 | 110 | 45·3 ± 1·2 |
| 60 | 47·2 ± 2·6 | 120 | 45·2 ± 1·2 |
| **Mean** (10–60 min) | 46·3 ± 1·0 | Mean (70–120 min) | 46·0 ± 0·5 |

### *Effect of different substrates on the endogenous respiration of the perfused rat heart*

Hearts were perfused with Krebs-bicarbonate medium containing 5 mM sodium acetoacetate, 5 mM acetoacetate + insulin 2 m-u./ml., 5 mM sodium succinate, and 20 mM sodium β-hydroxybutyrate, and the time course of the oxygen uptake at 37° C was measured over the first hour of perfusion (Table 4). As with other perfusion media, the $Q_{O_2}$ fell over the first 15–20 min, and then remained relatively steady. The mean $Q_{O_2}$ between

15 and 60 min of perfusion with media containing succinate or β-hydroxybutyrate was similar to that of hearts perfused with no added substrate, but that of hearts perfused with medium containing acetoacetate was significantly less than the control, although the difference was small (approx. 10%). Insulin had no effect on oxygen consumption in the presence of acetoacetate. The mean $Q_{O_2}$ of hearts perfused with succinate

TABLE 4. Effect of different substrates on the $Q_{O_2}$ of the rat heart perfused at 37° C

| Time of perfusion (min) | $Q_{O_2}$ (μl. $O_2$/mg dry wt./hr)* at 37° C with additions to the perfusion medium | | | |
|---|---|---|---|---|
| | 5 mM acetoacetate | 5 mM acetoacetate + Insulin 2 m-u./ml. | 5 mM succinate | 20 mM β-hydroxybutyrate |
| No. of expts.... | 7 | 6 | 4 | 7 |
| 5 | 38·5 ± 2·9 | 36·0 ± 2·8 | 41·3 ± 1·9 | 41·1 ± 1·9 |
| 10 | 36·8 ± 2·7 | 34·3 ± 2·2 | 40·1 ± 2·2 | 38·6 ± 1·6 |
| 15 | 34·7 ± 2·2 | 32·2 ± 2·0 | 37·6 ± 1·0 | 36·7 ± 1·5 |
| 20 | 33·3 ± 2·4 | 31·9 ± 1·7 | 36·2 ± 1·4 | 36·1 ± 1·5 |
| 30 | 34·0 ± 2·0 | 35·0 ± 1·5 | 37·2 ± 1·6 | 36·6 ± 1·9 |
| 40 | 34·0 ± 1·7 | 36·8 ± 2·1 | 37·9 ± 2·1 | 37·6 ± 2·0 |
| 50 | 33·9 ± 1·7 | 34·5 ± 1·5 | 36·8 ± 1·6 | 37·8 ± 1·8 |
| 60 | 34·1 ± 1·6 | 34·4 ± 1·2 | 34·2 ± 0·5 | 37·0 ± 2·1 |
| Mean (15–60 min) | 34·1 ± 0·7 | 34·1 ± 0·6 | 36·7 ± 0·5 | 36·9 ± 0·7 |

* Mean ± standard error of the mean.

showed a tendency to fall between 50 and 60 min, as sometimes occurred with hearts perfused in the absence of added substrate. The $Q_{O_2}$ of hearts perfused with acetoacetate or β-hydroxybutyrate remained constant for longer periods. Figure 2 shows that the $Q_{O_2}$, flow rate, and the heart rate of a heart perfused with 10 mM β-hydroxybutyrate can remain fairly constant over a period of 3 hr.

### *Effect of adrenaline on the oxygen consumption of the perfused rat heart*

In a previous paper (Fisher & Williamson, 1961) it was reported that adrenaline at a concentration of approximately $10^{-6}$ M greatly increased the flow rate and the oxygen uptake of the perfused rat heart. This effect has been confirmed, and the influence of the time of addition of adrenaline on the response of the $Q_{O_2}$ has been investigated in hearts perfused with nutrient-free medium, 8·3 mM glucose, and 8·3 mM glucose + insulin 2 m-u./ml. The results for the three perfusion media are shown in Table 6.

Adrenaline at the concentration used produces a maximum effect and almost doubles the $Q_{O_2}$. The rise in the $Q_{O_2}$ is however transient and the $Q_{O_2}$ returns to its original level in about 30 min. Table 6 shows that adrenaline can stimulate the $Q_{O_2}$ to a similar extent, both in the presence and

absence of glucose in the perfusion medium, but that, whereas in the presence of glucose the full adrenaline effect is obtained even after 2·5 hr, in the absence of nutrient in the medium the ability of the heart to respond to adrenaline with an increased $Q_{O_2}$ disappears between 30 and 55 min after the beginning of perfusion. Differences were also observed in the response to a second dose of adrenaline in the presence and absence of glucose. With hearts perfused in the absence of glucose a second similar

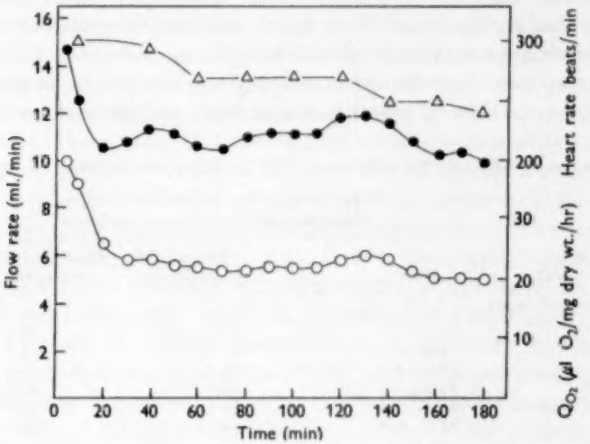



Fig. 2. Long-term maintenance of oxygen uptake in the presence of 10 mM β-hydroxybutyrate. The figure shows heart rate (beats/min, △), $Q_{O_2}$ (●) and coronary flow rate (ml./min, ○).

TABLE 5. Effect of adrenaline on the $Q_{O_2}$ of rat hearts perfused at 38·5° C. Adrenaline conc. $10^{-6}$ M (approx.)

| Perfusion medium | Time of adrenaline additions | $Q_{O_2}$ (µl. $O_2$/mg dry wt./hr) Before | Max. | Max. adrenaline effect on $Q_{O_2}$ | Duration of response (min) |
|---|---|---|---|---|---|
| Nutrient-free | 20 | 35 | 77 | +42 | 30 |
| | 30 | 38 | 65 | +27 | 25 |
| | 30 | 44 | 73 | +29 | 35 |
| | 55 | 28 | 28 | 0 | 0 |
| | 60 | 38 | 38 | 0 | 0 |
| 8·3 mM glucose | 60 | 36 | 75 | +39 | 30 |
| | 75 | 45 | 75 | +30 | 30 |
| | 125 | 38 | 59 | +21 | 15 |
| | 160 | 26 | 54 | +28 | 40 |
| 8·3 mM glucose + insulin 2 m-u./ml. | 60 | 45 | 65 | +20 | 25 |
| | 110 | 39 | 69 | +30 | 35 |
| | 120 | 47 | 75 | +28 | 40 |
| | 130 | 40 | 70 | +30 | 25 |
| | 140 | 34 | 55 | +21 | 30 |
| | 155 | 26 | 29 | + 3 | 10 |

dose of adrenaline added at 40 min, following a first dose at 20 min, produced a peak in the $Q_{O_2}$ about half as high as the first peak; but a second dose to another heart at 50 min following a first dose at 30 min had no effect on the $Q_{O_2}$. These experiments suggest that the ability of the heart to respond to adrenaline falls off after about 40 min. In another experiment glucose was added to the perfusate after 40 min, and adrenaline added at 75 min. A small rise in the $Q_{O_2}$ was obtained which was, however, much smaller than the maximum effect, and was of short duration. A second dose of adrenaline added to hearts perfused from the beginning with glucose produced a maximum effect similar to the first dose. Table 5 shows that the response to adrenaline is in every way similar, in hearts perfused with glucose, to that in hearts perfused with glucose and insulin.

TABLE 6. Effect of the addition of DNP on $Q_{O_2}$ of the perfused rat heart

| Time of perfusion (min) | $Q_{O_2}$ (μl./mg dry wt./hr)* at 35° C. Additions to the perfusion medium: None | Glucose 150 mg/100 ml. | Glucose 150 mg/100 ml. + insulin 2 m-u./ml. |
|---|---|---|---|
| No. of expts. | 3 | 4 | 7 |
| 5 | 32·3 ± 4·0 | 29·6 ± 3·0 | 33·9 ± 2·2 |
| 10 | 31·6 ± 4·2 | 28·6 ± 3·0 | 33·6 ± 2·2 |
| 15 | 31·7 ± 3·7 | 29·2 ± 2·4 | 33·6 ± 2·0 |
| 20 | 31·3 ± 3·9 | 29·7 ± 2·4 | 33·3 ± 2·0 |
| 25 | 31·4 ± 3·4 | 30·4 ± 2·5 | 32·7 ± 2·0 |
| 30 | 31·5 ± 4·4 | 30·5 ± 2·5 | 32·6 ± 1·8 |
| | DNP ($10^{-5}$ M) added at 30 min | | |
| 35 | 40·8 ± 1·4 | 38·2 ± 2·2 | 40·2 ± 1·2 |
| 40 | 42·2 ± 2·4 | 42·6 ± 3·8 | 47·2 ± 2·0 |
| 45 | 37·6 ± 1·9 | 42·7 ± 3·8 | 45·4 ± 1·7 |
| 50 | 35·7 ± 2·8 | 42·8 ± 3·8 | 45·4 ± 1·7 |
| 55 | 33·7 ± 2·8 | 42·8 ± 3·8 | 44·7 ± 1·6 |
| 60 | 31·5 ± 3·5 | 42·8 ± 3·8 | 44·3 ± 1·8 |

* Mean ± standard error of the mean.

*Effect of 2,4-dinitrophenol on the oxygen consumption of the perfused rat heart*

2,4-Dinitrophenol at a concentration of $10^{-5}$ M increases the $Q_{O_2}$ of perfused rat hearts by about 40%, without affecting the flow rate. The hearts continue to beat, but the heart rate falls to about 150 beats/min. Table 6 shows the effects of the addition of DNP at 30 min to hearts perfused at 35° C with nutrient-free medium, 8·3 mM glucose, and 8·3 mM glucose + insulin 2 m-u./ml. In the presence of glucose the elevated oxygen uptake is maintained, but in the absence of exogenous substrate the $Q_{O_2}$ falls to its original level after 30 min. Insulin has no effect on the response of the heart to DNP in the presence of glucose. Higher concentrations of DNP ($2 \times 10^{-5}$ M) caused the heart to stop beating and the oxygen uptake fell rapidly after an initial slight stimulation.

## DISCUSSION

It is clear from a study of Tables 1, 2 and 3 that the perfused rat heart can maintain a high rate of respiration for periods up to 90 min, and that the rate of the endogenous respiration is not influenced by the addition of glucose or insulin to the perfusion medium. The only observed effect of glucose is to prolong the steady-state rate of oxygen uptake of some of the hearts, and glucose with insulin is no more effective than glucose alone.

Bleehen & Fisher (1954) and Bronk & Fisher (1957) have found the glucose utilization of the perfused rat heart to be 12–15 mg/g dry wt./hr in the absence of insulin, and 44–48 mg/g dry wt./hr in the presence of insulin 2 m-u./ml. which is the concentration giving the maximum effect on glucose uptake. Fisher & Lindsay (1956) showed that only about one quarter of the glucose uptake in the presence of insulin 2 m-u./ml. was deposited as glycogen, and that very little lactate was formed. The glucose uptake in the absence of insulin is not sufficient to account for more than one third of the oxygen uptake, even if it is completely oxidized. The glucose uptake in the presence of insulin could account for all the oxygen uptake if it were completely oxidized. Nevertheless, insulin does not bring about an increase in oxygen consumption. Later work by one of us (J.R. W.) suggests that very little of the extra glucose used is in fact oxidized, so that the effect of insulin on the glucose metabolism of the heart is certainly not primarily that of increasing the general level of oxidative processes. It is important not to relate these results to the metabolism of the heart *in vivo* without taking into account that the isolated hearts used in these experiments are doing no work. Some factor not yet known must play a part in raising the level of oxidative metabolism to the level necessary to support the performance of external work, and it may well be that under the influence of this factor circulating glucose may become the primary food-stuff of the organ. This is suggested by the demonstration by Bing, Siegel, Ungar & Gilbert (1954) that glucose, rather than non-carbohydrate metabolites, is the major energy source of the heart *in vivo*.

It is interesting to note that the $Q_{O_2}$ of the perfused rat heart is considerably higher than that of incubated rat heart slices (Pearson, Hastings & Bunting, 1949), indicating that in these latter tissue preparations respiration is controlled by different factors from those which operate in the perfused, fully oxygenated, preparation, despite the fact the $Q_{10}$ value of 2·3 obtained in these studies agrees well with the figures of 2·0–2·2 observed by Fuhrman, Fuhrman & Field (1950) for rat heart slices incubated at temperatures up to 37·7° C. In this connexion it should also be noted that the oxygen uptake of rat heart slices is markedly increased

by substrates which have no effect on the perfused rat heart (Pearson *et al.* 1949). It seems unlikely, therefore, that attempts to investigate the regulation of cell metabolism based on preparations such as heart slices can have much relevance to the physiological problem.

The fact that the $Q_{O_2}$ can be increased by adrenaline and by DNP indicates that the failure to stimulate oxygen uptake with nutrients is not due to some technical imperfection of our preparation which limits its oxygen uptake. The results obtained with these agents in the presence of glucose indicate also that glucose can gain access to the site of glucose oxidation. If this were not so, it would be difficult to account for (*a*) the prolongation by glucose of the DNP effect in elevating oxygen uptake and (*b*) the preservation of the otherwise evanescent responsiveness to adrenaline by having glucose in the perfusate. Adrenaline and DNP both increase the total amount of oxygen consumed by hearts perfused with nutrient-free medium, and hence must stimulate the oxidation of endogenous metabolites. The increased oxygen uptake caused by DNP is not, however, maintained, and adrenaline is not effective in increasing the oxygen uptake if it is added towards the end of the first hour of perfusion, or if a second dose is given shortly after the first. Fisher & Williamson (1961) have shown that after 30 min perfusion with nutrient-free medium the glycogen content of the heart is reduced to about 8 mg/g dry wt. (expressed as glucose after correction for the non-utilizable apparent glycogen). The complete oxidation of this amount of glycogen requires 6 ml. of oxygen. A calculation of the oxygen used by hearts perfused with nutrient-free medium containing DNP (Table 5) shows that approximately 5·3 ml. extra $O_2$/g dry wt. is used during the period from 30 to 60 min. Similarly, on average, 6·8 ml. $O_2$/g dry wt. is used in excess of the normal oxygen uptake when adrenaline is added to hearts during the first 30 min. of perfusion. This extra oxygen cannot, however, all be used for the oxidation of glycogen, since nearly all the glycogen is used in any case by the end of 60 min perfusion with nutrient-free medium. Hence DNP and adrenaline must also stimulate oxidation of non-carbohydrate nutrients. The lack of a prolonged DNP effect, the abolition of the adrenaline effect, and the eventual fall in the oxygen uptake with nutrient-free medium, are all probably due to depletion of glycogen in the heart, and it is possible that oxidation of glycogen is necessary to replenish intermediates lost from the citric-acid cycle. When glucose is present in the medium these could be supplied by the oxidation of glucose, and the DNP effect, the adrenaline effect, and the steady rate of oxygen uptake could thus be maintained. Similarly, it is expected that adrenaline would also stimulate glucose uptake, although this is apparently not the case in isolated rat diaphragm (Walaas & Walaas, 1950). Randle & Smith (1958) found that

DNP stimulated glucose uptake by rat diaphragm, and it might be expected to have a similar effect in the perfused heart. The absence of a potentiating effect of insulin on the stimulation of the $Q_{O_2}$ by DNP and adrenaline strengthens the conclusion reached earlier that insulin is unlikely to be exerting a direct effect on the energy-producing mechanisms of the cell.

## SUMMARY

1. Glucose (8·3 mM), and glucose (8·3 mM) with insulin (2 m-u./ml.), are shown to have no effect on the oxygen consumption of the perfused rat heart.

2. In the absence of substrates in the perfusion medium the oxygen uptake of the rat heart falls after 40–90 min of perfusion, but can be maintained for up to 3 hr in the presence of nutrients.

3. The oxygen uptake of the rat heart is unaffected by the presence of 5 mM succinate or 20 mM $\beta$-hydroxybutyrate, but is slightly, though significantly, lowered in the presence of 5 mM acetoacetate or 5 mM acetoacetate + insulin 2 m-u./ml.

4. The $Q_{O_2}$ of the rat heart is markedly dependent on temperature, being $43{\cdot}2 \pm 0{\cdot}7(38)$ at 38·5° C and $32{\cdot}3 \pm 0{\cdot}6(25)$ at 35° C; giving a $Q_{10}$ of 2·3.

5. $10^{-5}$ M 2,4-dinitrophenol increases the oxygen uptake of the rat heart by about 40 % without affecting the flow rate. This stimulation of oxygen uptake is maintained in the presence of glucose, but disappears after 30 min if the perfusion medium is nutrient-free.

6. $10^{-6}$ M (approx.) adrenaline greatly increases the flow rate and the oxygen uptake of the rat heart. This effect is produced up to 160 min after the beginning of perfusion in the presence of glucose, but disappears between 30 and 55 min when the perfusion medium is nutrient-free.

7. It is concluded that insulin does not act by stimulating oxidative metabolism.

## REFERENCES

Bing, R. J., Siegel, A., Ungar, I. & Gilbert, M. (1954). Metabolism of the human heart. II. Studies on fat, ketone and amino acid metabolism. *Amer. J. Med.* **16**, 504–515.

Bleehen, N. M. & Fisher, R. B. (1954). The action of insulin in the isolated rat heart. *J. Physiol.* **123**, 260–276.

Bronk, M. S. & Fisher, R. B. (1957). The interaction of growth hormone and insulin in the perfused rat heart. *J. Physiol.* **136**, 435–448.

Creese, R., Scholes, N. W. & Whalen, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

Edson, N. L. (1935). Ketogenesis-antiketogenesis. I. The influence of ammonium chloride on ketone-body formation in liver. *Biochem. J.* **29**, 2082–2094.

Fisher, R. B. & Lindsay, D. B. (1956). The action of insulin on the penetration of sugars into the perfused heart. *J. Physiol.* **131**, 526–541.

Fisher, R. B. & Williamson, J. R. (1961). The oxygen uptake of the perfused rat heart. *J. Physiol.* **158**, 86–101.

FUHRMAN, G. J., FUHRMAN, F. A. & FIELD, J. (1950). Metabolism of rat heart slices, with special reference to effects of temperature and anoxia. *Amer. J. Physiol.* **163**, 642–647.

GEMMILL, C. L. & HAMMAN, L. (1941). The effect of insulin on glycogen deposition and on glucose utilization by isolated muscles. *Johns Hopk. Hosp. Bull.* **68**, 50–57.

KREBS, H. A. & EGGLESTON, L. V. (1945). Metabolism of aceto-acetate in animal tissues. *Biochem. J.* **39**, 408–419.

PEARSON, O. H., HASTINGS, A. B. & BUNTING, H. (1949). Metabolism of cardiac muscle: utilization of $^{14}C$ labelled pyruvate and acetate by heart slices. *Amer. J. Physiol.* **158**, 251–260.

RANDLE, P. J. & SMITH, G. H. (1958). Regulation of glucose uptake by muscle. 1. The effects of insulin, anaerobiosis and cell poisons on the uptake of glucose and release of potassium. *Biochem. J.* **70**, 490–500.

WALAAS, O. & WALAAS, E. (1950). Effect of epinephrine on rat diaphragm. *J. biol. Chem.* **187**, 769–776.

*J. Physiol.* (1961), **158**, *pp.* 113–131
*With 4 text-figures*
*Printed in Great Britain*



# PASSAGE OF PLASMA ALBUMIN INTO THE INTESTINE OF THE SHEEP

BY ROSA M. CAMPBELL, D. P. CUTHBERTSON, W. MACKIE, A. S. McFARLANE, A. T. PHILLIPSON AND SAOVANEE SUDSANEH*

*From the Rowett Research Institute, Bucksburn, Aberdeen and the National Institute for Medical Research, Mill Hill, London, N.W. 7*

(*Received* 1 *March* 1961)

Arising out of demonstrations that plasma albumin is present in the contents of the gastro-intestinal tract in some pathological states (Citrin, Sterling & Halstead, 1957; Freeman, 1958; Gordon, 1959; Schwartz & Jarnum, 1959) the possibility has been discussed that it escapes in smaller amounts into the lumen of the intestine in health (McFarlane, 1958*a*), and recently Holman, Nickel & Sleisenger (1959) claim to have demonstrated the presence of serum protein in the intestinal fluids of normal subjects, as well as in cases of hypoproteinaemia. Holman *et al.* (1959) also found serum protein in saliva and gastric juice of normal persons. Stimulation of gastric secretion with histamine diminished the concentration of serum protein in the gastric juice. While this might have been an enzymic effect, it could also have been due simply to dilution. A portion of the serum protein in the gastric juice could have been derived from swallowed saliva, but it is unlikely to be a significant source in the intestinal fluid.

In the course of studies on the distribution of $^{131}$I-albumin in extensive burns and at operations a high specific activity was observed in the stomach and duodenum and also in the upper part of the small intestine by Birke, Liljedahl & Plantin (1958) and Birke, Liljedahl, Plantin & Wetterfors (1959, 1960). It was found that after intravenous administration of $^{131}$I-albumin to normal subjects and to patients with carcinoma of the stomach labelled albumin appeared in the gastric juice. This occurred normally in such degree that a substantial part of the albumin break-down would appear to take place in the stomach and upper part of the small intestine (Birke, Gullberg, Liljedahl, Olhagen, Plantin & Wetterfors, 1960). Other substances of high molecular weight, such as dextran, may also be excreted via the gastric mucosa in man (Troell & Åberg, 1952). Several workers have found this mode of elimination as a likely one for albumin in

* International Atomic Energy Commission Fellow.

'exudative gastro-enteropathy' (Kimbel, Heinkel & Börner, 1956; Schwartz & Jarnum, 1959).

In a more recent investigation Ullberg, Birke, Ewaldsson, Hansson, Liljedahl, Plantin & Wetterfors (1960) showed that labelled plasma albumin collects to some degree in the small intestine of cats and mice and is discharged into its lumen. A certain part of the normal albumin break-down may, therefore, take place in the upper portions of the small bowel, which, together with the stomach, could well be the site of the main normal break-down of albumin.

It has also been shown in experiments involving washing of sections of the intestines of rabbits injected with either serum $^{131}$I-albumin or $^{131}$I-iodide that serum albumin passes from the circulation of rabbits into the duodenum (Armstrong, Margen & Tarver, 1960). If this type of transfer occurs throughout a considerable part of the gastro-intestinal tract, then the enzymic break-down of serum albumin so transferred could account for most of the serum albumin break-down which occurs *in vivo*.

Katz, Sellers & Golden (1960) could find no break-down of labelled human, rat and rabbit albumins when incubated with tissue slices of liver, kidney, spleen, lung, heart, muscle and skin of rat and rabbit: break-down of exogenous $^{35}$S biosynthetically labelled homologous albumin and globulin occurred only in slices of small intestine.

Taken as a whole the findings to date do not allow us to answer the question whether the plasma proteins are actively secreted into the intestine or appear by passive transudation. As the injection of histamine by Holman *et al.* (1959) did not increase the identifiable plasma proteins in the gastric or intestinal juices of man, it would appear that the proteins are not secreted although the result may be merely a reflexion of a simultaneous increase in enzymic activity.

Batty & Bullen's (1961) observations on sheep indicate that, with antitoxin as a marker, up to 1·34% of the serum concentration of the antitoxin can be found in the intestinal water. Antitoxin has the advantage of being readily recognized and quantitatively estimated, and degradation of this protein destroys its specific activity. Badawy, Campbell, Cuthbertson & Fell (1957) and Badawy, Campbell, Cuthbertson, Fell & Mackie (1958) have pointed out that the mode of inducing death may affect the integrity of the intestinal mucosa and that this may in turn complicate an interpretation of the analytical data. Fell (1961) has shown how such post-mortem shedding of the epithelium of the mucosa of the intestine may be prevented.

Protein may reach the lumen of the gastro-intestinal tract through the various digestive secretions and through the secretion of the goblet cells in the mucosa. Although the presence of many such activities indicates

that appreciable quantities of protein must enter the lumen of the gut, there have been few investigations on the nature or quantity of nitrogen involved and how far there may be a transference from the plasma via the lymph. Hogan (1957) has reported a definite addition of nitrogen to a washed-out loop of sheep's intestine, mainly in the form of protein. It may be that in Hogan's isolated loop and in Mosenthal's (1911) isolated portion of lower ileum of dog the source of the nitrogen found there was in part the proteins of lymph.

We report below experiments undertaken to investigate the possibilities of leakage or exudation of albumin into the digestive tract of the sheep, and for this purpose $^{131}$I-albumin was injected. Sterling (1951) has found good agreement between the disappearance rates of $^{131}$I-human albumin in the rabbit whether measured by radioactivity or by immunochemical methods. Cohen, Holloway, Matthews & McFarlane (1956) showed that $^{131}$I- and $^{14}$C-albumins had identical half-lives, and the view is now widely accepted that provided certain safeguards are observed in the labelling procedure (cf. McFarlane, 1956), $^{131}$I-proteins can behave in a fashion truly representative of the native proteins.

Following $^{131}$I-albumin injections man and most animals excrete practically all the label as iodide in the urine. In the male rat 2–3% is associated with protein in the urine (Campbell, Cuthbertson, Matthews & McFarlane, 1956). In some pathological conditions, however, 10–15% is excreted in the faeces, suggesting intestinal leakage of albumin into the digestive tract (Cattell & Norris, 1957; Gordon, 1959).

## METHODS

### *Electrophoresis*

Sheep serum was dialysed against a borate buffer (0·0115 M-$Na_2B_4O_7$ and 0·0155 M-$NaH_2PO_4$, pH 8·6) and fractionated by zone electrophoresis on a column of treated cellulose (Porath, 1956). The column measured 35 × 2·5 cm and was surrounded by a jacket through which water was circulated. Electrophoresis was carried out at 18 mA and 450 V for 46 hr. The eluate was collected in 4 ml. volumes and protein peaks located by measurement of the ultraviolet absorption at 280 mμ (Fig. 1). Albumin was concentrated by pressure dialysis at 4° C before labelling.

### *Iodination*

Albumin solutions were labelled with carrier-free $^{131}$I (supplied without reducing agent by the Radiochemical Centre, Amersham) after preliminary oxidation of sulphydryl groups using a technique described by one of us (McFarlane, 1958*b*). The protein solution was adjusted to pH 4·5 by addition of glycine buffer (7·2 g glycine dissolved in 96 ml. NaCl solution, 1·47 g/100 ml., and 12 ml. N-HCl). The acidified protein was mixed with unlabelled free iodine until the colour of free iodine appeared and the solution was then passed through an anion-exchange column of deacidite (Permutit and Co. Ltd). The pH of the effluent was then raised to 8·5 by addition of 0·1 N-NaOH and the desired amount of $^{131}$I in carrier iodine monochloride added. The solution was passed through a second similar deacidite column.

Less than 2% of the radioactivity in the column effluent was present in the supernatant after precipitation with 10% trichloroacetic acid (TCA). The mean ratio of iodine bound to albumin was always less than 1 g-atom/mole. The labelled proteins were diluted at once with an excess of unlabelled sheep serum.

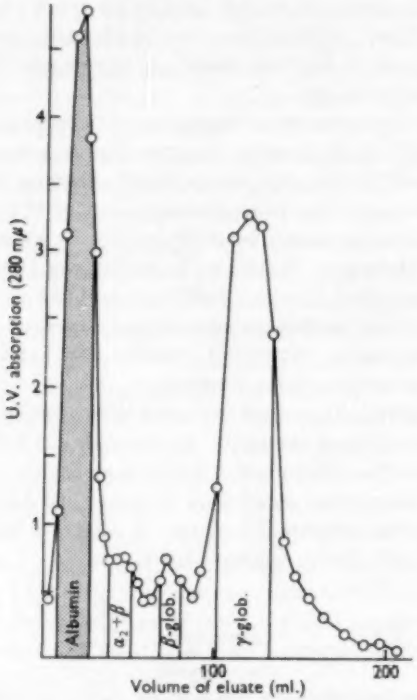



Fig. 1. Chromatography of sheep serum on carboxymethyl cellulose. Albumin, shown as a shaded area, was concentrated by pressure dialysis. For details, see text.

*Procedure*

All the sheep (Blackface wethers 1–2 yr old) were placed in metabolic cages a few days before the beginning of the experiment. The animals were fed twice daily at 10 a.m. and 6 p.m. on a diet of concentrates (linseed (4% N):oats (1·6% N), 2:1) and hay (1·6% N) or grass (2·4% N) as stated, and were given sodium iodide drinking water (25 mg NaI/l. (except in Expt. 1)) for 4 days before intravenous injection and throughout the entire experimental period.

In the experiments involving surgical techniques (for the insertion of cannulae or isolated loops), at least 4 weeks elapsed after the operation before the sheep were transferred to the cages for the isotope work.

*Surgical preparations*

| Expt. no. | Sheep no. | |
|---|---|---|
| 3 | 6 | Two Perspex cannulae were fitted in the duodenum, the first just caudal to the pylorus and the second at the iliac flexure after the entrance of the common bile and pancreatic duct. The third cannula was situated in the lower ileum |
| 4 | 7 | Isolated loop of the upper jejunum commencing at the iliac flexure, i.e. at the lower end of the duodenum (see Fig. 2) |
| 5 | 8 | |
| 6 | 9 | |
| 7 | 195 | Isolated loop of upper jejunum, 107 cm in length |
| 8 | 196 | Isolated loop of middle jejunum, 102 cm in length |
| 9 | 197 | Isolated loop of lower ileum, 63 cm in length |
| 10 | 194 | Isolated loop of upper jejunum, 71 cm in length |

*Collection of samples*

Plasma; blood samples were heparinized and centrifuged.
Urine; total 24 hr volume was measured and sample counted.
Faeces; the total 24 hr quantity was weighed and a known amount homogenized with 5% KOH and the final volume measured. A suitable sample was counted.
Cannulae samples (Expt. 3); the gut contents from each cannula were allowed to flow direct into a measuring cylinder.

*Isolated loop samples*

(*a*) Expts. 4 and 5. The loop was washed out thoroughly with physiological saline at 39° C, and the secretions or exudations were then allowed to flow direct into cylinders. The volumes produced over the stated intervals of time were recorded.
(*b*) Expts. 6–10. The loops were washed out as in (*a*) and then filled with a known volume of saline. This was allowed to remain in position over the stated time intervals. The fluid was then drained off into a measuring cylinder and the loop refilled with warm saline. The differential treatment of samples is recorded under each particular experiment when necessary. In Expts. 4–10 the flow of food along the remainder of the gut was maintained by connecting the 1st and 4th cannulae with plastic tubing.

*Detailed procedure of individual experiments*

*Experiment* 1. Sheep 1 and 2, 63 lb. (28·7 kg) and 59 lb. (27·2 kg) respectively, were given 200 g hay and 200 g concentrates twice daily. For 4 days before injection of $^{131}$I-albumin intravenously the drinking water contained 100 mg NaI/l. Plasma was obtained at regular intervals over the 21 days. Urine and faeces were collected at 24 hr intervals; in the case of sheep 2 for 21 days. As these sheep refused to eat, the iodide drinking water was suspected. Sheep 1 died on the 12th day. The 2nd sheep was then given untreated water to drink and various foodstuffs were offered to try to keep it alive. It eventually recovered.

*Experiment* 2. For 4 days before injection the drinking water contained 25 mg NaI/l. Sheep 3, 4, 318, 319 and 320, weighing 75, 70, 93, 100 and 92 lb. (34, 31·8, 42·2, 45·3 and 41·8 kg) respectively, were given 200 g grass and 50 g concentrates twice daily. Sheep 5, weighing 73 lb. (33·1 kg), was given 200 g hay and 50 g concentrates twice daily. Plasma, faeces and urine were sampled as in Expt. 1 (Fig. 3).

*Experiment* 3. Sheep 6 (75 lb., 34 kg) was given 200 g concentrates and 300 g dried grass twice daily. $^{131}$I-albumin was given intravenously. Plasma and cannulae samples were taken at regular intervals (Table 2). During the period immediately before and after the administration of labelled albumin five samples from each of the three cannulae were suitably diluted and cultured in four types of media, McConkey, Reinforced Clostridial Medium of

Hirsch & Grinstead (1954) and two media based on those used for culture of anaerobic rumen bacteria and containing casein or horse serum. During the period of administration of labelled albumin serial samples from the three cannulae were formolized and separated by differential centrifugation at 0° C into supernatant, small bacteria and larger bacteria and debris. The proportions of their fractions varied with the site of the sample.

*Experiment* 4. Sheep 7 (83 lb., 37·6 kg) was given 300 g grass and 200 g concentrates twice daily. The isolated upper jejunal loop was washed out thoroughly and after 0·5 hr the animal was injected intravenously with $^{131}$I-albumin. The secretions (intestinal contents from the distal end of the loop) and plasma samples were taken at the times stated after injection (Table 3).

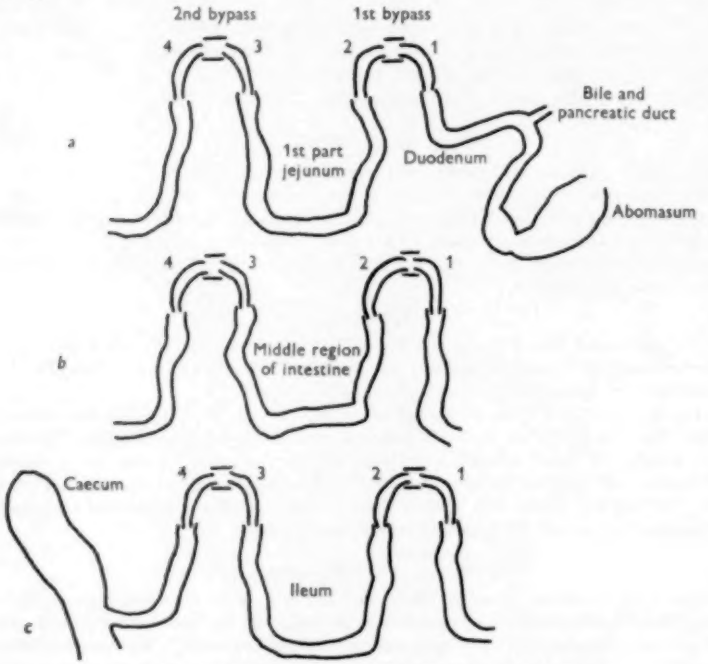



Fig. 2. Diagram showing positions of isolated loops in intestine.
*a*, Expts. 4, 5, 6, 7, 10; *b*, Expt. 8; *c*, Expt. 9.

*Experiment* 5. Sheep 8 (80 lb., 36·7 kg) was given 300 g hay and 200 g concentrates twice daily. The isolated lower duodenum–upper jejunal loop was washed out with saline and after 0·5 hr the animal was injected intravenously with $^{131}$I-albumin, and duplicate portions of intestinal contents and loop collections were taken at stated intervals. The intestinal and loop samples were measured for total radioactivity and then precipitated with TCA (final concentration 5 %) and the clear supernatant fluids measured for non-precipitable activity. After 2 hr the loop was reconnected into the intestine, and with the lower connexion as a cannula intestinal samples were taken daily for 5 days (Table 3).

*Experiment* 6. Sheep 9 (77 lb., 35 kg) was injected intravenously with $^{131}$I-albumin after the upper jejunal loop had been washed out with saline and refilled with 50 ml. saline. Loop samples were then taken at 1 hr and at six 2-hourly intervals (Table 4).

*Experiments* 7, sheep 195 (65 lb., 29·5 kg); 8, sheep 196 (69 lb., 31·4 kg) and 9, sheep 197 (60 lb., 27·3 kg). In each experiment $^{131}$I-albumin was injected intravenously and a plasma sample taken 5 min later. After 2 days the loop was isolated, washed out and a measured quantity of saline was left in position over six 2-hourly periods. The fluid drained off was divided into two samples (Table 5) thus: (1) protein precipitated with TCA (final concentration 5%); and (2) untreated sample.

*Experiment* 10, sheep 194 (60 lb., 27·3 kg). $^{131}$I-labelled sodium iodide was injected intravenously and plasma and loop samples were taken as in Expts. 7, 8 and 9 (Table 7).

## RESULTS

*Effect of iodide in the drinking water.* Experiment 1. Sheep 1 and 2 were given 0·01% NaI in the drinking water. This concentration is normally used to protect the thyroid of most laboratory animals and man when given $^{131}$I-albumin intravenously. The sheep, however, refused to eat and drink and became seriously ill. One of them died: the other recovered when the iodide level in the drinking water was reduced. This unusual behaviour was shown in the next experiment to be due to an unusually low kidney clearance rate for iodide. The results from this experiment have not been considered further.

*Catabolism of albumin by normal sheep.* Experiment 2. On the basis of the above experience experiments were continued with lower levels of NaI (0·0025%) in the drinking water. Sheep 3, 4, 318, 319, and 320 received intravenous injections of $^{131}$I-albumin (approximately 200 $\mu$c) and sheep 5 received 231 $\mu$c $^{131}$I-iodide. Results showed that sheep 5 excreted 21% of its total iodide pool per day compared with over 100% in man and various laboratory animals. This explains why 4 days were required for sheep 3 and 4 to achieve steady rates of iodide output in the urine (Fig. 3).

Catabolism of labelled albumin was unusual in several respects and we briefly draw attention to the following features as being of general interest. Albumin half-lives in the plasmas of the animals were very different, 336–680 hr, but in all cases corresponded approximately with catabolic rates determined from daily combined faecal and urinary excretions (4·4–10% plasma albumin catabolized per day) (Table 1).

After 12–15 days in all sheep the proportion of radioactivity in the plasma not precipitable with trichloroacetic acid, and the catabolic rates, began to rise in a manner not hitherto observed in other species (Fig. 3). An additional unique feature was the high and progressively increasing proportion of the total excreted activity appearing in the faeces. In the later stages nearly 20% of the excreted activity was in the faeces. Progressive re-utilization of iodotyrosine or of its break-down products by bacteria in the rumen is a possible explanation for this and for the apparently increasing catabolic rates.

*Experiment with cannulated sheep.* Experiment 3. $^{131}$I-albumin was injected into sheep 6, which had been provided with cannulae at the three stated levels in the gastro-intestinal tract. Table 2 shows the rate of appearance of radioactivity in the digesta from the three cannulae. When the semi-solid samples were centrifuged, most of the activity was in the supernatant fluid and was not precipitable with trichloroacetic acid. A few samples were exhaustively dialysed and lost most of their radio-activity in the process but a small proportion was clearly not dialysable, suggesting that faecal activity may have part of its origin above the duodenal level.

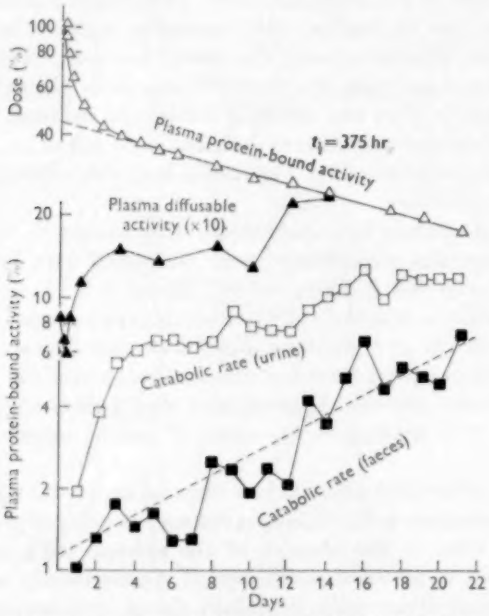



**Fig. 3. Turnover results obtained with $^{131}$I-labelled sheep albumin, Expt. 2.**

Although morphologically distinct bacteria could be seen in the samples from cannulae 1 and 2, little growth was found in the media inoculated from these samples, and much of this was due to sporing organisms which might not have been active in the intestine. As was to be expected, more microbial activity was demonstrated in the ileum.

Although the organisms cultured attacked the casein in the medium quite extensively, little break-down of the serum protein in the medium was found. Whether this can be correlated with the apparently negligible

TABLE 1. Plasma-albumin half-lives and catabolic rates in five normal sheep (Expt. 2)

| Sheep | | | | Plasma albumin | | | Proportion of catabolic rate due to faecal excretion | | Diffusible activity in plasma | |
|---|---|---|---|---|---|---|---|---|---|---|
| No. | Weight (lb.) | (kg) | $^{131}$I-alb. prepn. | Half-life (hr) | Extrapolated Y-intercept (%) | Catabolic rate* (%/day) | 100 hr | 400 hr | (%) | Time of max. (hr) |
| 3 | 75 | 34 | *A* | 678 | 44 | 4·4 | 7·5 | 16·5 | 1·0 | 60 |
| 4 | 70 | 31·8 | *A* | 375 | 45 | 7 | 23 | 42 | 1·5 | 75 |
| 318 | 93 | 44·2 | *B* | 336 | 45·5 | 10 | 6 | 9·5 | 1·15 | 170 |
| 319 | 100 | 45·3 | *B* | 504 | 44 | 8 | 9 | 12·5 | 1·2 | 260 |
| 320 | 92 | 41·8 | *B* | 513 | 44 | 7 | 16 | 10 | 1·2 | 220 |

* 24 hr urinary and faecal radioactivity as a percentage of the total radioactivity in the plasma at the mid point of the time interval.
Preparations *A* and *B* were used respectively in experiments made in February 1958 and October 1960.

TABLE 2. Activities in plasma and digesta samples of sheep 6 (Expt. 3), which received 177 $\mu$c $^{131}$I-albumin intravenously at zero time

| | Plasma | | Digesta from | | |
|---|---|---|---|---|---|
| Time | Activity (m$\mu$c/ml.) | Fraction TCA-precipitable (%) | First part of duodenum (m$\mu$c/ml.) | Lower end of duodenum (m$\mu$c/ml.) | Ileum (m$\mu$c/ml.) |
| 5 min | 88 | 99·3 | 0·17 | 0·34 | — |
| 30 min | — | — | 0·25 | 0·80 | 0·03 |
| 1 hr | 75 | 99·5 | 0·77 | 1·34 | — |
| 4 hr | 61·5 | 99·4 | 1·12 | 1·30 | 0·30 |
| 10 hr | 47 | 99·2 | 1·56 | 1·43 | 1·61 |
| 24 hr | 37 | 98·8 | 1·47 | 1·44 | 0·42 |
| 48 hr | 33 | 97·5 | 1·97 | 1·63 | 0·50 |

uptake of labelled albumin, or its derived amino acids, into the micro-organisms at these sites is impossible to decide from this experiment.

Further experiments were required to investigate the possibility that some or all of the non-dialysable faecal activity in sheep injected with this labelled albumin might be due to iodide incorporated irreversibly into bacteria and other micro-organisms.

*Experiments with sheep having isolated jejunal loops.* Experiments 4, 5 and 6. $^{131}$I-albumin was injected into three sheep, each having an isolated loop of jejunum, with the following results:

(*a*) In Expts. 4 and 5, where a comparison was made, the activity per millilitre in the clear fluid collected from the temporarily isolated upper jejunal loops was found to rise to more than 10% of the value in the animal's plasma at the same time (Table 3). No plasma activities were recorded in Expt. 6 but the level of activity found in the loop (Table 4) was of the order noted in Expt. 4. Practically all this activity was protein-bound. On the other hand, the activity per millilitre of the 'free flowing' intestinal digesta, as obtained at the junction of cannulae 1 and 4, was of a lower order, increased slowly and as in the previous experiments was mainly iodide.

Two hours after the injection of the albumin in sheep 8 (Expt. 5) the loop was reconnected. The activity in samples of digesta from cannula 3 (Fig. 2*b*), that is, after they had now passed through the loop, was reduced and was mainly iodide, corresponding to the finding in the earlier samples (up to 2 hr) of intestinal digesta. A comparison of the properties of this earlier material with the digesta obtained after reconnexion was made as follows: Samples precipitated with 15% trichloroacetic acid (final concentration 5%) were centrifuged and both deposits containing about a quarter of the total activity were dialysed against running water. Only 20% of the activity remained in the sac, indicating that not more than 5% of the total activity in the digesta could be protein-bound. This could not easily be investigated further because of the inevitable bulk of TCA-insoluble material in the digesta.

Since both samples behaved in the same way, it appeared that either the contribution of succus entericus protein-bound activity represents only a small proportion of the total activity in the lumen, or the arrival of proteolytic enzymes in the now reconnected loop rapidly leads to hydrolysis of labelled succus protein within it. This experiment was repeated after injecting $^{131}$I-iodide instead of albumin (Expt. 10, sheep 194).

(*b*) The loop of sheep 9 (Expt. 6) was washed through with 50 ml. saline solution at the end of each period of collection from the cannula. During the course of the experiment bile and (apparently) gut contents entered the loop. The wash fluids in all cases had about one-third the

TABLE 3. Experiment using isolated duodenal–jejunal loops

| Expt. no. | Sheep no. | Approx. dose of $^{131}$I-albumin injected ($\mu$c) | Times after injection at which samples were taken | Plasma | | Jejunal loop fluid | | Intestinal contents (Fig. 2*a*): Lower end of duodenum, cannula 1 | | Intestinal contents (Fig. 2*a*): Reconnected loop, cannula 3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | (m$\mu$c/ml.) | Fraction TCA-precipitable (%) | (m$\mu$c/ml.) | Fraction TCA-precipitable (%) | (m$\mu$c/ml.) | Fraction TCA-precipitable (%) | (m$\mu$c/ml.) | Fraction TCA-precipitable (%) |
| 4 | 7 | 206 | 5 min | 100 | 98·2 | — | — | 0·50 | — | — | — |
| | | | 30 min | — | — | — | — | 1·07 | — | — | — |
| | | | 1 hr | 85 | 98·5 | 11·1 | 96·2 | 1·26 | — | — | — |
| | | | 5½ hr | 62 | 98·6 | 15·0 | 96·5 | 1·60 | — | — | — |
| | | | 10 hr | 48·5 | 98·6 | 11·5 | 94·8 | 2·08 | — | — | — |
| | | | 23 hr | 40 | 98·1 | 12·7 | 93·8 | 3·20 | — | — | — |
| | | | 46 hr | 37 | 98·15 | 14·0 | 91·8 | 4·11 | — | — | — |
| 5 | 8 | 350 | 5 min | 148 | — | — | — | — | — | — | — |
| | | | 30 min | 138 | — | 2·22 | 88 | 2·75 | 31 | — | — |
| | | | 1 hr | 133 | — | 7·58 | 92 | 3·48 | 29 | — | — |
| | | | 2 hr | 125 | — | 15·1 | 97 | 3·74 | 28 | — | — |
| (After reconnecting loop at duodenal end and collecting daily from distal end (cannula 3) | | | 24 hr | — | — | — | — | — | — | 4·11 | 30 |
| | | | 48 hr | — | — | — | — | — | — | 3·83 | 23·5 |
| | | | 72 hr | — | — | — | — | — | — | 3·30 | 24 |
| | | | 96 hr | — | — | — | — | — | — | 3·98 | 21·5 |
| | | | 120 hr | — | — | — | — | — | — | 2·84 | 26 |

TABLE 4. Experiment using isolated duodenal–jejunal loop

| Expt. no. | Sheep no. | Approx. dose of $^{131}$I-albumin injected ($\mu$c) | Times after injection at which samples were taken (hr) | Saline in loop (ml.) | Fluid recovered from loop (ml.) | Jejunal loop fluid: (m$\mu$c/ml.) | Jejunal loop fluid: Fraction TCA-precipitable (%) | Washings: No. | Washings: m$\mu$c in 3 ml. | Washings: Fraction TCA-precipitable |
|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 9* | 215 | 1 | 50 | 40 | 0·72 | 30·0 | — | — | — |
| | | | 2 | 50 | 41 | 0·73 | 26·4 | 1 | 0·627 | 43·2 |
| | | | 4 | 50 | 32·5 | 1·12 | 32·5 | 2 | 1·098 | 49·6 |
| | | | 6 | 50 | 36 | 1·09 | 34·5 | 3 | 0·949 | 51·1 |
| | | | 8 | 50 | 47 | 1·70 | 32·9 | 4 | 2·430 | 52·4 |
| | | | 10 | 50 | 37 | 1·91 | 36·9 | 5 | 2·186 | 49·3 |
| | | | 12 | 50 | 60 | 1·96 | 21·7 | — | — | — |

* This sheep was subsequently found to have a duodenal-jejunal anastomosis resulting from coalescence of adjacent parts of small intestine.

activity per millilitre of the sample obtained before washing, and half of this was precipitable with TCA, indicating that digestion had proceeded, though not so far as in the normal intestinal contents. At this time it was observed that activities in TCA precipitates were soluble in 1% TCA-ethanol, which is consistent with the presence of $^{131}$I-albumin.

The use of radioactive media and radiography showed that, as suspected, union between the lumen of the loop and the end of the intestine had occurred, as appeared from observations made during the experiment, and post-mortem examination confirmed this finding, although the union was small and adjacent to the cannulae. The rising activities were therefore due to increasing amounts of iodide coming from the stomach. Despite this occurrence the experiment confirms hydrolysis of the proteins in the intestinal contents at that level.

*Experiments* 7, 8 *and* 9. This series was designed to facilitate the identification of albumin in the intestinal contents and therefore only fluid from isolated loops was used. $^{131}$I-albumin was injected 48 hr before isolating the loop, which was then washed out every 2 hr with 50 ml. saline solution. The results (Table 5) are essentially similar in all three animals and confirm the presence of non-precipitable activity in the loops. The proportion of this activity diminished as the sampling distance from the pylorus increased, possibly owing to incorporation into micro-organisms in the ileum.

Six loop samples were dialysed under pressure until the protein concentration was 1–2% and the protein was then submitted to electrophoresis in starch gel, using the technique described by Smithies (1959), along with samples of serum taken at the same time. The stained gels were photographed, and Fig. 4 shows a typical result demonstrating the presence of an albumin-like protein and very little else in the loop fluid. Serial strips of gel were cut out and the starch hydrolysed by boiling with 1% HCl for 3 hr (3 ml. for each 2 g wet gel). The hydrolysates were centrifuged and proteins in the supernatant fluid precipitated with TCA. The results in Table 6 show significant radioactivity to be present only in the albumin regions and demonstrate that specific radioactivities of albumins in the plasma and loop samples were similar.

*Experiment* 10, in which sheep 194 was injected with $^{131}$I-sodium iodide, and which is in essence a control to sheep 8 and others, was carried out to decide whether any of the protein-bound activity in the loop samples in the previous experiments could have originated from $^{131}$I-iodide released by catabolism of albumin and recombined into succus or other free intestinal proteins. A dose of $^{131}$I-iodide (calc. 800 $\mu$c) was therefore chosen, which would give activities in loop samples of the same order as non-protein activities in the previous experiments with $^{131}$I-albumin

TABLE 5. Experiments using isolated jejunal loops

| Expt. no. | Sheep no. | $^{131}$I-albumin injected ($\mu$c, approx.) | Times after injection at which samples were taken min | hr | Plasma (m$\mu$c/ml.) | Saline in loop (ml.) | Loop fluid: Recovered from loop (*a*) (ml.) | Loop fluid: Activity (*b*) (m$\mu$c/ml.) | Loop fluid: Fraction TCA-precipitable (*c*) | Loop fluid: Protein activity in total fluid = $a \times b \times c$ (m$\mu$c) |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 195 | 800 | 5 | | 437 | — | — | — | — | — |
| | | | 20 | | 344 | — | — | — | — | — |
| | | | | 48 | 105 | — | — | — | — | — |
| | | | | 50 | 93·1 | 50 | 47·5 | 3·60 | 0·88 | 150 |
| | | | | 52 | 98·4 | 50 | 46 | 3·67 | 0·89 | 150 |
| | | | | 54 | 96·7 | 50 | 50 | 2·72 | 0·85 | 116 |
| | | | | 56 | 99·5 | 50 | 50 | 1·33 | 0·66 | 44 |
| | | | | 58 | 104 | 50 | 50 | 1·77 | 0·76 | 67 |
| | | | | 60 | 96·0 | 50 | 60 | 1·70 | 0·73 | 37 |
| 8 | 196 | 192 | 5 | | 121 | — | — | — | — | — |
| | | | | 48 | 33·5 | — | — | — | — | — |
| | | | | 50 | 32·5 | 75 | 55 | 0·48 | 0·55 | 14·5 |
| | | | | 52 | 32·5 | 75 | 44 | 0·84 | 0·67 | 26 |
| | | | | 54 | 32·1 | 75 | 59 | 0·82 | 0·67 | 32·5 |
| | | | | 56 | 31·8 | 75 | 46 | 0·58 | 0·62 | 16·5 |
| | | | | 58 | 30·7 | 75 | 51 | 0·76 | 0·67 | 26 |
| | | | | 60 | 31·3 | 75 | 32 | 0·86 | 0·61 | 16·7 |
| 9 | 197 | 324 | 5 | | 142 | — | — | — | — | — |
| | | | | 48 | 39·0 | — | — | — | — | — |
| | | | | 50 | 38·7 | 100 | 134 | 0·41 | 0·49 | 27 |
| | | | | 52 | 38·5 | 100 | 139 | 0·67 | 0·45 | 42 |
| | | | | 54 | 37·3 | 100 | 104 | 0·52 | 0·30 | 16 |
| | | | | 56 | 35·1 | 100 | 119 | 0·47 | 0·34 | 18 |
| | | | | 58 | 34·9 | 50 | 54 | 0·71 | 0·51 | 21 |
| | | | | 60 | 35·9 | 100 | 90 | 0·73 | 0·64 | 42 |

(Table 7). In every case all the activity in the loop sample was found in the TCA supernatant showing that the sheep cannot use iodide to produce protein-like compounds in the jejunum. This finding, and that noted in Expt. 3, suggest that diffusible radioactivity liberated by the break-down of albumin is being incorporated into bacteria, either anterior to the pylorus (reticulo-rumen most probably) or more likely in the caecum and colon.

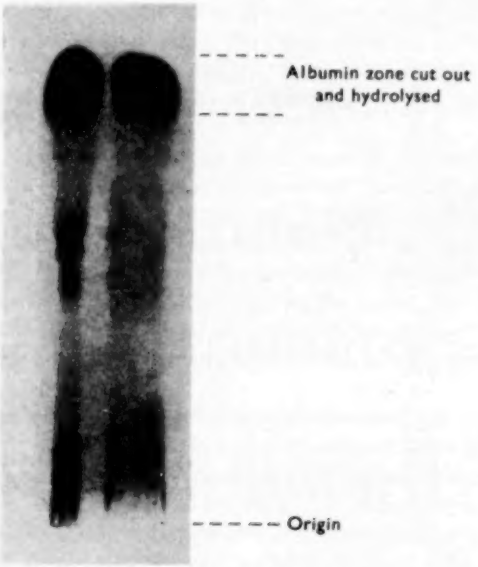



**Fig. 4.** Starch gel electrophoresis of plasma (left pattern) and 'loop fluid' (right) proteins obtained at the same time.

From the histological observations made on the tissues of the loops of Expts. 7–10 it appeared that the loops were healthy and free from necrosis or inflammation. The mucosae were essentially normal in appearance but there was generally an hypertrophy of the lymphatics. Adhesions were present in most animals and could be a cause of such lymphatic enlargement.

## DISCUSSION

From these experiments identification of plasma albumin as a constituent of the contents of the jejunum in the sheep appears to be established both on electrophoretic and on specific-activity bases. While protein-like

precipitable radioactivity was demonstrated in test samples of the digesta from injected sheep which were accustomed to the presence of cannulae over many months and were in good health, this represented but a small proportion of the total radioactivity of the samples, which was mainly due to $^{131}$I-iodide secreted, presumably, by the salivary glands and gastric mucosa. When cannulae connexions were rearranged in such a way as to

TABLE 6. Comparison of radioactivity of albumins isolated from simultaneous plasma and loop samples from injected sheep

| Expt. no. | Sheep no. | Times after injection at which samples were taken (hr) | Radioactivity (mμc/g albumin) | |
|---|---|---|---|---|
| | | | Plasma | Loop fluid |
| 7 | 195 | 48 | 1210 | 976 |
| | | 50 | 1200 | 964 |
| 8 | 196 | 50 | 838 | 692 |
| | | 60 | 644 | 542 |
| 9 | 197 | 58 | 844 | 650 |
| | | 60 | 808 | 580 |

TABLE 7. Plasma and loop-fluid activities following injection of $^{131}$I-iodide into sheep 194 (Expt. 10). None of the activity in the loop samples was precipitable with trichloroacetic acid

| Time | Plasma (mμc/ml.) | Loop samples (mμc/ml.) |
|---|---|---|
| 5 min | 31·1 | — |
| 48 hr | 3·7 | 1·57 |
| 50 hr | 3·4 | 1·52 |
| 52 hr | 3·2 | 1·13 |
| 54 hr | 2·3 | 0·91 |
| 56 hr | 1·9 | 0·97 |
| 58 hr | 3·0 | 1·07 |
| 60 hr | 3·0 | — |

TABLE 8. Experiments using $^{131}$I-albumin

| Expt. no. | Sheep no. | $^{131}$I-albumin injected intravenously (μc) | Plasma (mμc/ml.) | Plasma volume |
|---|---|---|---|---|
| 2 | 3 | 226 | 151* | 1·50 |
| | 4 | 201 | 120* | 1·68 |
| | 318 | 172 | 96* | 1·79 |
| | 319 | 172 | 88* | 1·96 |
| | 320 | 172 | ~ 90* | ~ 1·91 |
| 3 | 6 | 177 | 88† | 2·00 |
| 4 | 7 | 206 | 100† | 2·06 |
| 5 | 8 | 350 | 148† | 2·37 |
| 7 | 195 | 800 | 437† | 1·83 |
| 8 | 196 | 192 | 121† | 1·59 |
| 9 | 197 | 324 | 142† | 2·28 |

* At 10 min; † at 5 min.

isolate a loop of jejunum, the clear succus entericus collected from this loop in 2 hr was equivalent in radioactivity and protein content to a ten-fold dilution of the plasma at the same time, and practically no iodide was present. Electrophoresis of this fluid using the high resolution of starch gel showed it to contain mainly albumin having a specific radioactivity approaching that in the plasma at the same time. Washing out the loop with saline solution did not increase the measurable radioactivity, suggesting that we are concerned here with a physiological secretion or exudation of albumin, and not a reaction to the saline and washing-out procedure used.

It is not possible to correlate the data obtained from loop samples with the measured catabolism of albumin in the same sheep on any quantitative basis. This arises from two facts: (*a*) that we can only guess at the contribution which the remaining (non-cannulated) section of the intestine made to the over-all albumin leakage, and (*b*) the albumin identifiable in loop samples represents only that portion of albumin which has resisted proteolytic digestion within the loop, although our evidence suggests that digestion, if it occurs therein, is very limited. Nevertheless, it is evident from the large fall in protein-bound activity per millilitre of intestinal fluid, which resulted from reconnecting the loop at 2 hr in Expt. 5 (Table 3), that digestion occurs in the loop on a substantial scale when stomach contents are passing through the loop in a physiological manner. Total activities recovered in two-hour loop washings from sheep 196 in the period 50–60 hr averaged 22 m$\mu$c, and 28 m$\mu$c in sheep 197 (cf. Table 5) based on measured plasma or loop albumin specific activities of approximately 800 m$\mu$c/g (cf. Table 6). These values correspond to approximately 28 and 35 mg respectively of plasma albumin secreted into the lumen in 2 hr. Larger amounts of albumin appeared in the lumen of sheep 195 in the interval up to 54 hr but were not sustained in subsequent washings. It appears, therefore, that only about one tenth of the total albumin known to be catabolized by the sheep in a given interval can be found unchanged in the isolated jejunal loop. The most that can be said on the quantitative aspects of this investigation is that substantial amounts of albumin can be recovered from the intestinal contents and that these do not exceed the amounts catabolized daily. This work, therefore, gives general support to the experiments of Armstrong *et al.* (1960) with rabbits, which indicated that the main site of break-down of plasma albumin is in the intestine.

These observations are also of particular interest when we consider the work of Nasset, Schwartz & Weiss (1955), who examined the total nitrogen recovered from various parts of the small intestine of the dog at death 1½ hr after a test meal, and noted that the level tended to remain constant in the animal regardless of the protein in the test meal. Nasset (1957) found it

difficult to escape the conclusion that the ingestion of a meal, whether or not it contained any protein, results in the transfer of appreciable material into the lumen of the small intestine. He considered that the hydrolytic enzymes and the mucoproteins of the digestive juices, together with desquamated cells of intestinal mucosa—the last being continually added to—and possibly other sources of protein, may provide enough endogenous protein which, on digestion, 'dilutes' the amino-acid mixture in the intestine sufficiently to obscure any peculiarity in amino-acid pattern of the food protein. Nasset put forward the view that the results of the mixing of amino acids in the lumen of the intestine may be to regulate rather closely the relative concentrations of the amino acids available for absorption. It is to be noted here that the technique used by Nasset *et al.* (1955) to obtain the samples of digesta for analysis would lead, we believe, to the kind of excessive shedding of intestinal epithelium described by Badawy *et al.* (1957, 1958) as occurring in the sheep and small laboratory animals. Fell (1961) has shown how such post-mortem desquamation may be prevented.

Our data (cf. Table 1) show a maximum catabolism of 8 g albumin/day (10 % of the plasma albumin) in the normal sheep. The N intake (Expt. 2) was some 12·8 g/day. If we assume on the basis of Hogan's (1957) finding that some 75 % of this would actually pass into the duodenum, the contribution of albumin will only amount to approximately 13 % of the nitrogen entering the small intestine. The contribution of the mucoproteins, digestive enzymes, cellular debris, etc. may be more substantial but has still to be determined. In the sheep microbial protein is of a high biological value and the advantages of balancing the amino acids in the digesta through secretions etc. is not so obvious as would be the case in non-ruminants.

SUMMARY

1. Intravenously administered $^{131}$I-plasma albumin was catabolized very slowly by the sheep, which also was found to have poor ability to excrete iodide. Sodium iodide at a concentration of 0·01 % in the drinking water was toxic: no untoward effect resulted from a concentration of 0·0025 %. Following catabolism of the labelled protein increasing proportions of the excreted radioactivity appeared in the faeces.

2. Protein-bound radioactivity was present in digesta obtained from cannulae in the duodenum and jejunum along with much larger amounts of $^{131}$I-iodide.

3. The sheep cannot use intravenously injected $^{131}$I-iodide to produce protein-bound radioactivity in the digesta or faeces.

4. Fluids from isolated loops of jejunum of sheep which had received $^{131}$I-serum albumin intravenously contained substantial amounts of

protein identified as plasma albumin on the bases of electrophoretic behaviour and specific radioactivity.

5. Quantitative aspects of these observations are discussed in relation to the site of normal catabolism of plasma albumin and to the possible role which this passage of albumin into the small intestine, at a maximum of 8 g/day, may play in supplementing the quota of endogenous protein being added to the food and microbial protein in process of being digested and absorbed. The maximum contribution of plasma-albumin N appears to amount only to some 13% of the nitrogen passing into the duodenum of the sheep on this particular type of ration.

We wish to thank Dr P. N. Hobson and Dr T. H. Blackburn for separating and examining the microbial fractions, Mr B. F. Fell for the histological examinations and Miss M. Grieveson for measuring the radioactivities.

## REFERENCES

ARMSTRONG, F. B., MARGEN, S. & TARVER, H. (1960). Plasma protein. VII. Site of degradation of serum albumin. *Proc. Soc. exp. Biol., N.Y.*, **103**, 592–596.

BADAWY, A. M., CAMPBELL, R. M., CUTHBERTSON, D. P., FELL, B. F. (1957). Changes in the intestinal mucosa of the sheep following death by humane killer. *Nature, Lond.*, **180**, 756–757.

BADAWY, A. M., CAMPBELL, R. M., CUTHBERTSON, D. P., FELL, B. F. & MACKIE, W. S. (1958). Further studies on the changing composition of the digesta along the alimentary tract of the sheep. 1. Total and non-protein nitrogen. *Brit. J. Nutr.* **12**, 367–383.

BATTY, I. & BULLEN, J. J. (1961). The permeability of the sheep and rabbit intestinal wall to antitoxin present in the circulation. *J. Path. Bact.* (in the Press).

BIRKE, G., GULLBERG, R., LILJEDAHL, S.-O., OLHAGEN, B., PLANTIN, L.-O. & WETTERFORS, J. (1960). Studier av albumin läckage i ventrikeln. *Nord. med.* **64**, 1179.

BIRKE, G., LILJEDAHL, S.-O. & PLANTIN, L.-O. (1958). Albumin metabolism in burns damage studied by albumin labelled with $^{131}$I. *Nord. med.* **60**, 963.

BIRKE, G., LILJEDAHL, S.-O., PLANTIN, L.-O. & WETTERFORS, J. (1959). Role of the stomach in the metabolism of albumin. *Nord. med.* **62**, 1741–1744.

BIRKE, G., LILJEDAHL, S.-O., PLANTIN, L.-O. & WETTERFORS, J. (1960). Albumin catabolism in burns and following surgical procedures. *Acta chir. scand.* **118**, 353–366.

CAMPBELL, R. M., CUTHBERTSON, D. P., MATTHEWS, C. M. & MCFARLANE, A. S. (1956). Behaviour of $^{14}$C and $^{131}$I-labelled plasma proteins in the rat. *Int. J. appl. Rad. Isotopes*, **1**, 66–84.

CATTELL, W. R. & NORRIS, T. ST M. (1957). Idiopathic hypercatabolic hypoproteinaemia. *Proc. R. Soc. Med.* **50**, 514–516.

CITRIN, Y., STERLING, K. & HALSTEAD, J. A. (1957). The mechanism of hypoproteinaemia associated with giant hypertrophy of the gastric mucosa. *New Engl. J. Med.* **257**, 906–912.

COHEN, S., HOLLOWAY, R. C., MATTHEWS, C. & MCFARLANE, A. S. (1956). Distribution and elimination of $^{131}$I and $^{14}$C-labelled plasma proteins in the rabbit. *Biochem. J.* **62**, 143–154.

FELL, B. F. (1961). Cell shedding in the epithelium of the intestinal mucosa: fact and artefact. *J. Path. Bact.* **81** (in the Press).

FREEMAN, T. (1958). In discussion of paper by FOWLER, J. F. and MILES, J. M., 'Rapid protein breakdown in idiopathic hypoproteinaemia'. *Radioaktive Isotope in Klinik und Forschung*, **3**, 311.

GORDON, R. S. (1959). Exudative enteropathy. Abnormal permeability of the gastrointestinal tract demonstrable with labelled polyvinylpyrrolidone. *Lancet*, **276**, 325–326.

HIRSCH, A. & GRINSTEAD, E. (1954). Methods for the growth and enumeration of anaerobic spore-formers from cheese, with observations on the effect of nisin. *J. Dairy Res.* **21**, 101–110.

HOGAN, J. P. (1957). The removal of nitrogen at different levels from the alimentary tract of the sheep. *J. Physiol.* **139**, 25*P*.

HOLMAN, H., NICKEL, W. F. & SLEISENGER, M. H. (1959). Hypoproteinaemia antedating intestinal lesions, and possibly due to excessive serum protein loss into the intestine. *Amer. J. Med. Sci.* **27**, 963–975.

KATZ, J., SELLERS, A. L. & GOLDEN, S. (1960). Plasma albumin catabolism in tissue slices. *Proc. Soc. exp. Biol., N.Y.*, **103**, 807–811.

KIMBEL, K. H., HEINKEL, K. & BÖRNER, W. (1956). Über die Herkunft des Eiweisses im Magensaft Untersuchungen mit markiertem Humanalbumin. *Ärztl. Wschr.* **11**, 602–607.

MCFARLANE, A. S. (1956). Labelling of plasma proteins with radioactive iodine. *Biochem. J.* **62**, 135–143.

MCFARLANE, A. S. (1958*a*). Métabolisme des protéines plasmatiques et syndrome néphrotique. *Brux. méd.* **38**, 1669.

MCFARLANE, A. S. (1958*b*). Efficient trace-labelling of proteins with iodine. *Nature, Lond.*, **182**, 53.

MOSENTHAL, H. O. (1911). Observations on the succus entericus. *J. exp. Med.* **13**, 319–327.

NASSET, E. S. (1957). Role of the digestive tract in the utilization of protein and amino acids. *J. Amer. med. Ass.* **164**, 172–177.

NASSET, E. S., SCHWARTZ, P. & WEISS, H. V. (1955). The digestion of proteins *in vivo*. *J. Nutrit.* **56**, 83–94.

PORATH, J. (1956). Methodological studies of zone-electrophoresis in vertical columns. 1. Fractionation in cellulose powder columns of substances of low molecular weight exemplified by amino acids and related compounds. *Biochim. biophys. acta*, **22**, 151–175.

SCHWARTZ, M. & JARNUM, S. (1959). Gastrointestinal protein loss in idiopathic (hypercatabolic) hypoproteinaemia. *Lancet*, **276**, 327–330.

SMITHIES, O. (1959). An improved procedure for starch-gel electrophoresis: Further variations in the serum proteins of normal individuals. *Biochem. J.* **71**, 585–587.

STERLING, K. (1951). The turnover rate of serum albumin in man as measured by $^{131}$I-tagged albumin. *J. clin. Invest.* **30**, 1228–1237.

TROELL, L. & ÅBERG, B. (1952). The carbohydrate content of the gastric juice after intravenous administration of partly hydrolysed, bacterial dextran to hospital cases. *Scand. J. clin. Lab. Invest.* **4**, 355–362.

ULLBERG, S., BIRKE, G., EWALDSSON, B., HANSSON, E., LILJEDAHL, S.-O., PLANTIN, L.-O. & WETTERFORS, J. (1960). The role of the gastrointestinal tract in the elimination of serum albumin. *Acta med. scand.* **167**, 421–425.

*J. Physiol.* (1961), **158**, *pp.* 132–143
*With 6 text-figures*
*Printed in Great Britain*

# AN INVESTIGATION INTO THE EXTERNAL SECRETION OF THE PANCREAS IN SHEEP

By D. F. MAGEE*
*From the Rowett Research Institute, Bucksburn, Aberdeen*

(*Received* 3 *March* 1961)

The external pancreatic secretion of simple-stomached animals, such as the dog and man, has been extensively studied during the past century (Thomas, 1950). In the ruminant, on the other hand, only a few studies have been conducted on the intestinal phase of digestion. The extensive fermentative digestion in these animals and the continuous flow of gastric contents to the duodenum suggested that the regulation of secretion of digestive juices into the duodenum might not conform to the pattern recognized in dogs. In addition, little information is available on the enzymic content of sheep pancreatic juice. For these reasons the experiments described here were undertaken.

## METHODS

Seven Blackfaced sheep were prepared, under Nembutal anaesthesia (pentobarbitone; Abbott Laboratories), with Thomas-type cannulae according to the method of Hill & Taylor (1957), with slight modification in that the gall bladder was anastomosed directly to the mid jejunum. This rendered unnecessary duodenal transection and re-anastomosis, required by the original method. Blackfaced sheep were found more satisfactory than other available breeds for operation because of their shorter rib cage. This meant that the point of entry of the common bile and pancreatic duct into the duodenum was more readily accessible than in the other breeds of sheep available. Each of the seven sheep had rumen and abomasal cannulae in addition to the duodenal cannula.

To collect pancreatic juice the duct was cannulated in the conscious animal and the juice allowed to flow into a graduated test tube fixed to the animal's side. The tube was changed and the volume of its contained juice measured every 15 min. In all samples amylase activity was estimated and the total output per fifteen minutes was calculated. In the following text amylase activity refers to the total output in each period, except where stated otherwise. In many samples chloride and bicarbonate were estimated, the former by electrometric titration with $AgNO_3$ (Sanderson, 1952) and the latter with the Van Slyke manometric apparatus. In every instance in the following text bicarbonate and chloride levels are expressed as concentrations.

Amylase was estimated by Stein & Fischer's (1958) modification of Sumner's (1925) method. The starch substrate was incubated with appropriately diluted juice for 3 min at 35° C. In all instances amylase activity is expressed as mg maltose/3 min at 35° C.

* John Simon Guggenheim Fellow, Department of Pharmacology, University of Washington, School of Medicine, Seattle 5, Washington, U.S.A.

The abomasal cannula allowed drainage of the abomasum when required and thus decreased or prevented the entry of abomasal juice into the duodenum. In addition, substances in solution were introduced into the duodenum through a tube which could be pushed through the abomasal cannula past the pyloric sphincter. Solutions were also introduced into the duodenum through the Thomas cannula. In Table 1 are listed most of the solutions and drugs used.

*Intraduodenal solutions.* Abomasal and rumen contents were obtained from the experimental or other specially prepared sheep. The obviously particulate material was removed by filtration through several layers of gauze.

TABLE 1. Effect of some substances and procedures on the volume of pancreatic juice secreted and on its amylase activity. Amylase activity is expressed as the mean of the total activity per 15 min collection

| | Mean volume (ml./15 min) | | Mean amylase activity (mg maltose) | | | |
|---|---|---|---|---|---|---|
| | Before | After | Before | After | No. of sheep | No. of expts. |
| Fasting 24–48 hr | 3·1 | 2·1 | 100 | 43* | 5 | 5 |
| Opening abomasal cannula | 3·15 | 2·4 | 103 | 50* | 7 | 11 |
| Introduction into the duodenum of: | | | | | | |
| Abomasal content, pH 2·5 | 1·5 | 2·5* | 40 | 60* | 5 | 7 |
| Dialysate of abomasal content, pH 2·5 | 1·83 | 2·58* | 59 | 116* | 7 | 11 |
| Dialysed abomasal content, pH 2·5 | 1·60 | 2·46* | 161 | 222 | 6 | 6 |
| Fatty acid 55 mM, pH 2·6 | 1·70 | 2·50* | 68 | 101* | 6 | 6 |
| Fatty acid 55 mM, pH 4·6 | 2·25 | 2·60 | 63 | 96 | 3 | 3 |
| Casein hydrolysate 5 % | 1·70 | 2·50 | 216 | 302* | 5 | 5 |
| Rumen liquor | 1·60 | 2·10 | 110 | 154 | 6 | 7 |
| HCl 0·1 N | 1·82 | 2·92* | 117 | 212* | 7 | 16 |
| Olive oil | 1·9 | 2·2 | 105 | 114 | 4 | 4 |
| Oleic acid | 2·4 | 2·3 | 78 | 94 | 6 | 7 |
| Pilocarpine (intravenous) | 2·00 | 4·40* | 71 | 182* | 6 | 6 |
| Abomasal inflation | 2·3 | 2·6 | 105 | 131 | 6 | 7 |

* Difference significant at $P = 0{\cdot}02$ level.

The dialysate of abomasal content was prepared by dialysing filtered abomasal content against an equal volume of water for 36 hr. Three changes of abomasal content were used against the same distilled water, allowing 12 hr for each. The same abomasal content was then dialysed against running tap water for 24 hr. In both cases the final pH was brought to 2·5 with HCl or NaOH.

The olive oil used was that sold for culinary use and the oleic acid was chemically pure. Only 10 ml. of either was used, 3 ml. by way of the Thomas cannula and 7 ml. by way of the abomasal cannula. Casein hydrolysate and peptone were used as 5 % solutions in water, as the Difco preparations.

The fatty acid solutions listed contained acetic, butyric and propionic acids either alone or mixed with appropriate concentrations of their salts to raise the pH of the final solutions from 2·6 to 4·6. In both the pH 2·6 or 4·6 solutions the concentration of the acids or acids plus salts was 55 mM, but the final solution was brought to isotonicity with NaCl. The ratio of the acid radicals to each other in both solutions was 7:2:1 for acetate, butyrate and propionate respectively.

Hydrochloric acid was used, usually as an 0·1 N solution. In some experiments where an attempt was made to correlate the pH of the solutions with the secretory response of the pancreas 0·2 N-HCl also was employed. In experiments of this sort (Table 2), 0·08 M citrate

buffer brought to isotonicity with NaCl was used for pHs above 2·6. The ratio of citric acid to its sodium salt was altered to bring the final solution to the required pH.

*Intravenous preparations.* Pilocarpine nitrate (0·01–0·1 mg/kg), secretin (Boots and Lilly) dissolved in water (5–22 u./animal) and pancreozymin (Boots) dissolved in saline (5–22 u./animal) were administered intravenously. In all the experiments involving injection of secretin or pilocarpine nitrate or the introduction of solutions into the duodenum the abomasal cannula was left open for at least 1 hr to obtain resting values for pancreatic secretion. The cannula was left open throughout the remainder of the experiment.

In all experiments in which solutions were introduced into the abomasum, except those using olive oil or oleic acid, a total volume of 40 ml. was introduced into the duodenum in 15 min; usually 30 ml. from the abomasum and 10 ml. through the Thomas cannula. About 1/3 of this volume was given initially and the remainder slowly during the subsequent 10 min. It was found that at least three 15-min collection periods were necessary before the rate of secretion of pancreatic juice returned to its resting value and another solution could be tested.

TABLE 2. A typical experiment showing the relation of pH of the solutions introduced into the duodenum to pancreatic amylase secretion. Amylase is expressed as the total activity per 15 min

| pH of solutions | Amylase |
|---|---|
| 7 | 228 |
| 4·5 | 384 |
| 3·5 | 348 |
| 2·6 | 476 |

To distend the abomasum a thick-walled rubber balloon attached to a catheter was pushed through the abomasal cannula and inflated with approximately 400 ml. of air. With this volume resistance greater than that due to the balloon alone could be felt.

All the procedures outlined above were carried out on four sheep. Of the remaining three animals, one lacked only the olive oil experiment. All the sheep were given intraduodenal fatty acid solution, but only three received this at pH 4·6. The sheep were housed and fed in loose pens. The diet was of hay and meal consisting of crushed oats and linseed meal in the proportion of 1:2.

## RESULTS

*Effect of fasting.* It was found that fed sheep with the abomasal cannula closed had an average secretory volume of 3·1 ml./15 min, containing amylase activity estimated at 100 mg of maltose. Both the volume secreted and the total amylase activity were reduced by fasting for 24 hr or more (Table 1). Even after a 48-hr fast secretion continued, but at a reduced rate (mean 0·9 ml./15 min). Similar reductions in the volume of juice secreted and its amylase content were observed if the abomasal cannula was allowed to drain for 60 min. It was noted that in all fed animals, with the abomasal cannula closed, the pH of the duodenal chyme obtainable through the Thomas cannula was between 2·5 and 3·5.

*Effect of abomasal contents.* The introduction of filtered abomasal contents, obtained from the same or other sheep, into the duodenum significantly increased both the volume and amylase of the secreted juice. In eleven

experiments abomasal dialysate was introduced into the duodenum and resulted in a significant increase in the output of amylase and in the volume of pancreatic juice secreted. The mean volume increased from 1·83 to 2·58 ml. and the amylase from 59 to 116 mg of maltose. Dialysed abomasal content, brought to the same pH as the dialysate used above, produced a significant increase in volume, but a variable amylase increase.

*Effect of pure foodstuffs and rumen liquor.* Solutions at pH 2·6 containing acetic, propionic and butyric acids increased the secretion of pancreatic juice and amylase. Solutions at pH 4·6 produced a much smaller volume increase, 0·35 ml., as opposed to 0·8 ml. in the experiments with solutions at pH 2·6. The mean amylase increase, however, was equal in both sets of experiments.

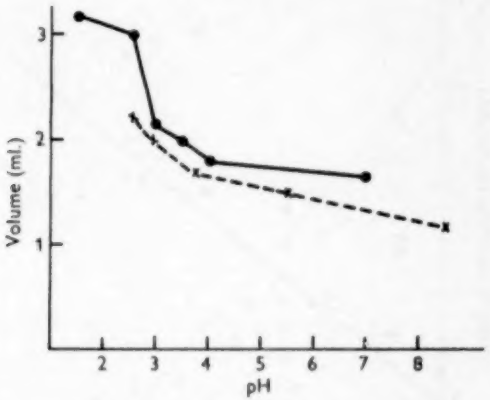



Fig. 1. The relationship between the volume of pancreatic juice secreted and the pH of solutions introduced into the duodenum. O—O Sheep 1; x - - - - x Sheep 2.

Rumen liquor, at pH 7, was introduced into the duodenum and produced equivocal responses. Casein hydrolysate in 5% solution produced an equivocal volume response but significantly increased the amylase content of the juice from 216 to 302 mg of maltose. The introduction of 5% peptone, of olive oil or of oleic acid was without effect.

*Effect of pH.* Figure 1 and Table 2 show one of the most notable findings of the experiments, namely, that both the volume of pancreatic juice and output of amylase activity vary inversely with the pH of the solutions introduced into the duodenum (correlation coefficient ($r$) for volume and pH $= -0{\cdot}534$; $P < 0{\cdot}03$ for four experiments). Citrate buffer was used to prepare the solutions for introduction except below pH 2·6 when HCl was used. The introduction of equal volumes of saline or of citrate buffer at pH 7 did not significantly alter the rate of pancreatic secretion.

*Abomasal distension.* In eight experiments on six sheep the abomasum was distended by introducing 400 ml. of air into a balloon which had been inserted through the abomasal cannula. No consistent pancreatic response resulted.

*Secretin and pancreozymin.* Figure 2 shows that following the intravenous injection of secretin in doses of from 5 to 20 u. the increase in the volume of pancreatic juice was linearly related to the logarithm of the dose administered. Table 3 suggests that not only does secretin increase the volume of juice, but also the amylase activity. Calculations from control and experimental data in which pancreatic secretion was stimulated either by HCl or by secretin indicate that the volume response and

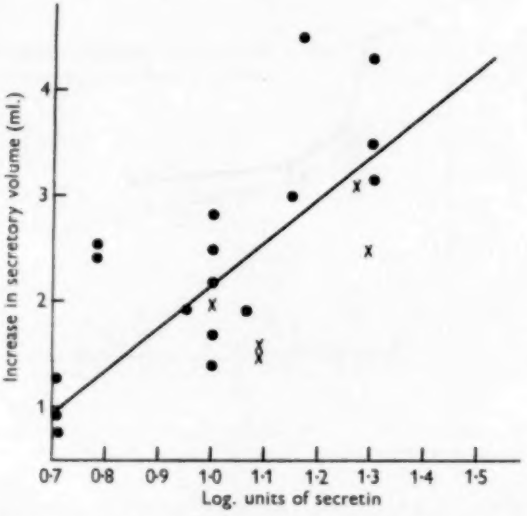



Fig. 2. The relationship between the dose of secretin and the increase in volume from the resting level. Regression line calculated from data obtained from seven sheep, $y = 3{\cdot}58x - 1{\cdot}45$, $n = 23$. Correlation coefficient $r = 0{\cdot}69$; $P < 0{\cdot}01$. Mean of controls from which differences calculated = 1·55 ml. × Boots secretin; ● Lilly secretin.

TABLE 3. The effect of secretin on the volume of pancreatic juice secreted and on its amylase content

| Secretin (u.) | Volume (ml.) | Total amylase (mg maltose) | Concentration (mg maltose/ml.) |
|---|---|---|---|
| 0 | 2·5 | 55 | 22 |
| 20 | 6·7 | 67 | 10 |
| 20 | 5·2 | 114 | 22 |
| 20 | 5·5 | 105 | 19 |
| 0 | 2·4 | 74 | 32 |

the amylase activity of the juice were directly related. A highly significant correlation coefficient ($r = 0{\cdot}78$; $P < 0{\cdot}01$) was obtained from 41 observations (Fig. 3).

The bicarbonate concentrations of the pancreatic juice always increased following the injection of secretin. In fact, in every experiment, except those in which secretion was stimulated by the injection of pilocarpine,

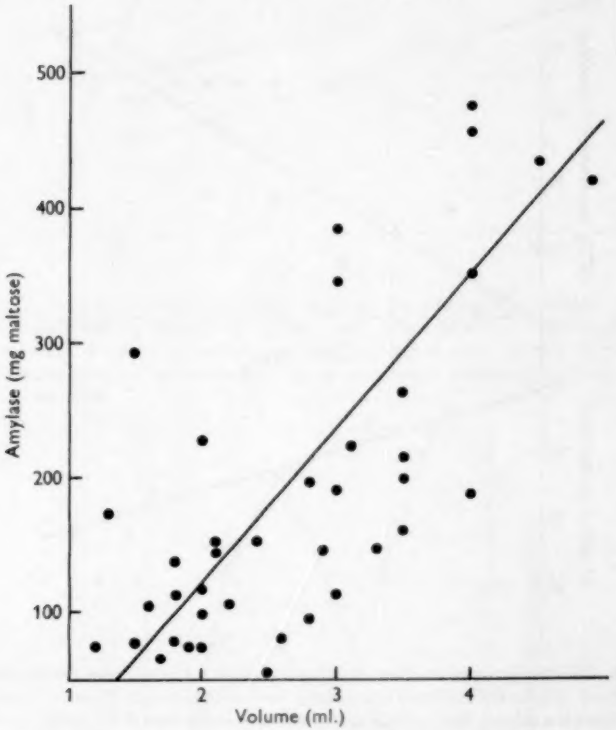



Fig. 3. Data from 41 experiments showing the relationship between the volume of juice secreted and the amylase activity per 15 min from the unstimulated gland and after stimulation by injected secretin or the introduction of 0·1 N-HCl into the duodenum. Equation for regression: $y = 114x - 105$; correlation coefficient, $r = 0{\cdot}81$, $P < 0{\cdot}01$, $n = 41$.

the increments in bicarbonate concentration and in the volume of juice were significantly related ($r = 0{\cdot}88$; $P < 0{\cdot}01$). Bicarbonate and chloride concentrations varied inversely. A significant relationship between chloride decrement and the volume response ($r = -0{\cdot}56$; $P < 0{\cdot}02$) was also found (Fig. 4).

*Pilocarpine.* In six experiments in six animals the mean increase in the volume of pancreatic juice following the injection of pilocarpine was 120% and the amylase activity output per 15 min increased by 158% (Table 1), but the bicarbonate and chloride concentrations remained unchanged (Figs. 5 and 6). In Fig. 6 it will be seen that if pilocarpine is injected when

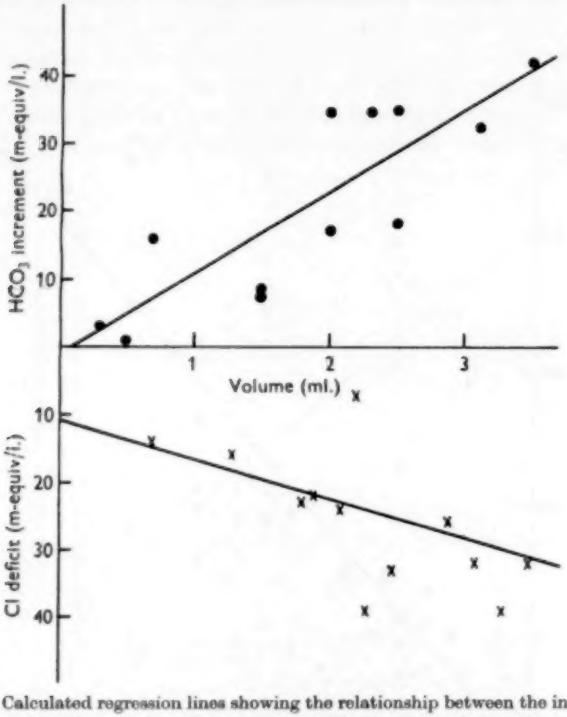



Fig. 4. Calculated regression lines showing the relationship between the increment in volume of juice secreted and the changes in bicarbonate and chloride concentration following stimulation by injecting secretin, introduction of 0·1 N-HCl into the duodenum or by feeding the sheep. ×—× chloride; ●—● bicarbonate. Chloride, $y = -10{\cdot}3 - 5{\cdot}9x$, $n = 15$; correlation coefficient, $r = -0{\cdot}56$; $P < 0{\cdot}02$. Bicarbonate, $y = 12{\cdot}2x - 1{\cdot}4$, $n = 14$; correlation coefficient, $r = 0{\cdot}88$; $P < 0{\cdot}01$. Mean of the controls from which the differences were calculated: chloride, 122 m-equiv/l.; bicarbonate = 25 m-equiv/l.

both the volume of juice and its bicarbonate concentration are falling after secretin the volume rises but the bicarbonate concentration continues to fall. The bicarbonate concentration of the juice is artificially high after the first injection of pilocarpine, because this sample contains juice remaining in the ducts and cannula from the previous collection period.

In general, chloride concentrations in the unstimulated juice were higher than those in dogs and man, ranging from 100 to 120 m-equiv/l. while bicarbonate concentrations were lower, ranging from 10 to 35 m-equiv/l. In dogs, at secretory rates of pancreatic juice of 0·75 ml./15 min,

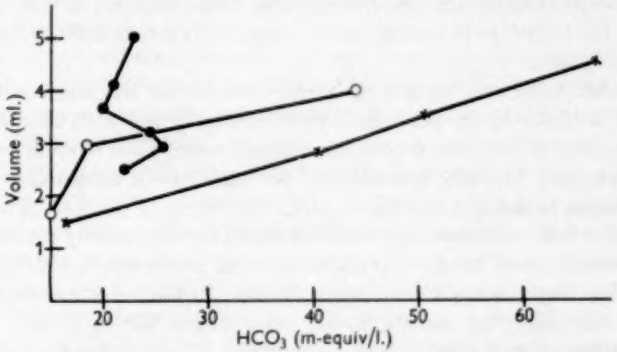



Fig. 5. Data from three experiments showing the relationship between volume and bicarbonate concentration in pancreatic juice following stimulation with intravenous secretin and pilocarpine and the introduction of acid of varying concentration into the duodenum. ●—● pilocarpine experiment; ○—○ acid; ×—× secretin.

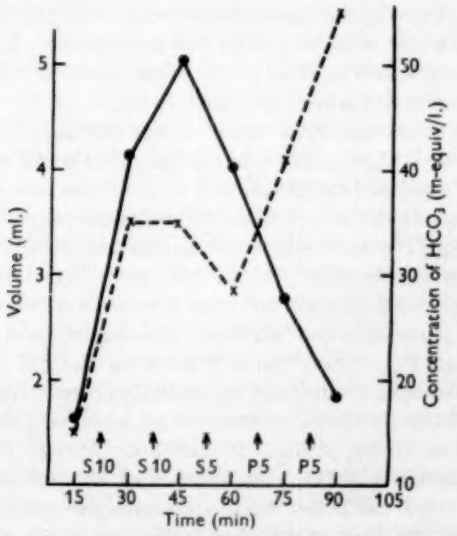



Fig. 6. Data from an experiment showing the effect of injecting secretin and pilocarpine on the volume of juice secreted and its bicarbonate concentration. S10 = 10 u. secretin; P5 = 5 mg pilocarpine. ×– – –× volume; ●—● bicarbonate.

the corresponding figures were 90 m-equiv/l. for chloride and 50 m-equiv/l. for bicarbonate (Bro-Rasmussen, Killman & Thaysen, 1956).

Because of the variation in resting values, it was necessary to base most calculations on the changes from observed resting values in each experiment, rather than on the absolute figures. This variation in resting value may be due to failure to remove all the stimuli of various intensities present in the intestine.

In addition to this variation, it was found that the concentration of amylase activity in the pancreatic juice gradually fell with time after the operation and did not rise even if the animals were rested from experiments for a fortnight. The amylase activity per millilitre of unstimulated juice, for example, in sheep 6 when first used, was 54 mg of maltose. A fortnight later, after four experiments in each of which a progressively smaller value was obtained, only 22 mg of maltose/ml. was produced. A similar phenomenon has been observed by Taylor (1958). In his experiments, amylase output declined progressively during experiments lasting 30 hr.

The effect of pancreozymin was equivocal. This may be due to the fact that several of the animals collapsed, even after slow injection, which suggests a profound drop in blood pressure.

## DISCUSSION

It is of value to compare the present results with previous results from dogs. The basal secretion after a 48 hr fast in these sheep had a mean value of 0·9 ml./15 min, which is greater than that found in the fasting dog in which it may be immeasurable (Babkin, 1950).

The reaction of the duodenal contents was one of the most interesting findings of the investigation since in our animals the pH was never above 3, and in other duodenal-fistula animals in which the bile was not diverted it never approached the values found in the duodenal contents of the dog following feeding. The abomasal contents entering the duodenum in sheep have pH values of this order (R. W. Ash, unpublished observation), but they are not apparently neutralized until they are well beyond the common opening of the pancreatic and bile duct. This finding, and the relationship demonstrated in Fig. 1 and Table 2 between the pH of the duodenal contents and the volume and amylase activity, respectively, suggest that the reaction of the duodenal contents is an important regulator of pancreatic secretion in the sheep. It has been found (Pincus, Thomas, Hausman & Lackman, 1948) that the pH of the duodenal contents of dogs and man must fall below 4·5 to stimulate pancreatic secretion. This is rarely, if ever, attained in dogs but is the rule in the sheep.

The high acidity of the duodenal contents poses two questions, namely, what prevents the sheep's duodenum from ulcerating and how do the

pancreatic digestive enzymes, all of which have pH optima of 7 or above, act in the duodenum? Sheep amylase is inactive below pH 4·5 (Magee, unpublished observation) but it resists peptic digestion (Stein & Fischer, 1958) and may act in the jejunum, where the contents have a higher pH. Proteolytic enzymes are, however, much more delicate and may conceivably be destroyed.

In the sheep, although volume changes in pancreatic secretion can be easily elicited by acid in the duodenum, by secretin and by other stimuli, these changes are, as a rule, much smaller than those seen in the dog. The largest response to secretin obtained in the present study from a 60 kg sheep was 6·7 ml., while from dogs 1 ml./kg can easily be obtained (Bro-Rasmussen *et al.* 1956). The electrolyte content of the pancreatic juice of sheep was notable in that at comparable rates of secretion the concentration of chloride was higher and the concentration of bicarbonate lower than in the juice of the dog. The direct relationships between the increments in volume and bicarbonate is seen in both species, except after pilocarpine stimulation in sheep (Figs. 5 and 6). If secretory rates, however, are expressed per unit of body weight they are seen to be very much lower in the sheep than in the dog. The latter with stimulation may secrete 0·08 ml./kg/min, while the most obtained under similar circumstances from sheep is 0·0067 ml./kg/min. Dogs secreting at such low proportional rates have electrolyte concentrations only slightly different from those found in sheep (Fig. 4), i.e. about 90 m-equiv/l. of chloride and 60 m-equiv/l. of bicarbonate (Bro-Rasmussen *et al.* 1956). There are no data on bicarbonate secretion in pancreatic juice following pilocarpine administration in dogs, since parasympathomimetic substances alone do not increase volume (Lin & Ivy, 1957). In the sheep pilocarpine was a powerful volume stimulant (Figs. 4 and 5). These data have led to the speculation that the mechanism by which pilocarpine stimulates the volume of juice secreted is different from that of secretin and other volume stimulants and that, with these agents, the change in volume is secondary to the change in bicarbonate. Further interpretation of the data is hampered by the fact that, because of variability in the control figures, calculations have all been based on increments rather than that on absolute quantities.

The amylase output, per collection period, of the sheep's pancreas was directly related ($r = 0{\cdot}78$; $P < 0{\cdot}01$) to the volume of pancreatic juice secreted (Fig. 3). Because of the size of the animals and the scarcity of secretin it was not possible to administer the hormone by continuous intravenous infusion, but the possibility of the effect being due to 'wash out' was reduced by giving repeated single doses (Table 3). When these were kept constant there was little reduction in the total output or concentration of amylase. In fact, the amylase concentration was usually

lower after the first injection than during subsequent periods and, in addition, the concentration of amylase and the total quantity secreted varied with the dose of secretin during repeated administrations.

TABLE 4. Ratio between the increase in amylase activity (%) and the increase in volume secreted (%) following the introduction of various substances into the duodenum

| Solution | Ratio |
|---|---|
| Abomasal filtrate | 2·92 |
| Abomasal filtrate dialysed | 0·47 |
| Abomasal filtrate dialysate | 2·10 |
| Fatty acid 55 mM, pH 2·6 | 2·20 |
| Fatty acid 55 mM, pH 4·6 | 3·20 |
| HCl 0·1 N | 1·90 |
| Secretin | 1·00 |
| Pilocarpine | 1·32 |

The question arises whether or not the secretion of amylase, when various foodstuffs and solutions were introduced into the duodenum, was secondary to the volume change. Table 4 shows that there is no constant ratio between the volume and amylase changes. This is especially clear in the fatty acid experiments, where in three animals fatty acid mixtures at pH 4·6 produced a smaller volume increment, but an increment in juice amylase activity equal to that produced by fatty acid solutions at pH 2·6. It is likely, therefore, that in the sheep as in the dog the quantity of enzyme secreted can vary independently from the volume of juice secreted. Abomasal contents and fatty acid solutions appeared to exert a greater effect on amylase secretion than the other stimuli used, and a part of the activity of the abomasal contents was due to the presence of a dialysable component, possibly of an acidic nature. It should be noted that the osmotic pressures of both the dialysed abomasal content and the dialysate were less than normal, i.e. about 85% of normal in the case of the dialysate and about 40% of normal in the dialysed content. Since neither the dialysed abomasal content nor isotonic saline increased the amylase output, it is unlikely that the increased juice amylase output evoked by the dialysate was due to its electrolyte deficiency.

In dogs and man gastric distension regularly produces an increase in the volume of juice secreted and an increase in its protein content (White, Lundh & Magee, 1960). The sheep's pancreas, however, did not respond to abomasal distension. In view of the way in which abomasal activity is integrated with that of the other compartments of the ruminant stomach (Phillipson, 1939), it is unlikely that distension is as usual an occurrence as in simple-stomached animals where it often follows every meal.

SUMMARY

1. Thomas-type pancreatic fistulae have been constructed in seven sheep.

2. The normal fed sheep secreted 3·1 ml./15 min of juice containing amylase activity equivalent to 100 mg of maltose according to the method used.

3. Both fasting (48 hr) and the absence of abomasal contents in the duodenum reduced secretion and amylase by approximately 50%.

4. The introduction into the duodenum of abomasal contents, a dialysate of abomasal contents, a 55 mM fatty acid solution or 0·1 N-HCl increased both volume and amylase. Five % casein hydrolysate increased only amylase significantly. Secretin and pilocarpine increased both volume and amylase.

5. The pH of the duodenal chyme was believed to be an important regulator of pancreatic activity.

6. Bicarbonate concentration increased directly as volume and chloride fell in every instance investigated, except after pilocarpine. Pilocarpine was without effect on either.

7. Balloon distension of the abomasum was without effect on secretory volume or amylase.

I wish to thank Dr D. P. Cuthbertson for the use of the facilities of the Institute and L. Vowles and E. Rae for valuable assistance and also Eli Lilly and Co for secretin.

REFERENCES

Babkin, B. P. (1950). *Secretory Mechanisms of the Digestive Glands*, 2nd ed., p. 197. New York: Paul Hoeber.

Bro-Rasmussen, F., Killman, S. & Thaysen, J. H. (1956). The composition of pancreatic juice as compared to sweat, parotid, saliva and tears. *Acta physiol. scand.* **37**, 97–113.

Hill, K. J. & Taylor, R. B. (1957). Collection of pancreatic juice from the conscious sheep. *J. Physiol.* **139**, 26*P*.

Lin, T. M. & Ivy, A. C. (1957). Relation of secretin to the parasympathetic mechanism for pancreatic secretion. *Amer. J. Physiol.* **189**, 361–368.

Phillipson, A. T. (1939). The movements of pouches of the stomach of sheep. *Quart. J. exp. Physiol.* **29**, 395–415.

Pincus, I. J., Thomas, J. E., Hausman, D. & Lackman, P. O. (1948). Relationship between pH of the duodenal content and pancreatic secretion. *Proc. Soc. exp. Biol., N.Y.*, **67**, 497–501.

Sanderson, P. H. (1952). Potentiometric determination of chloride in biological fluids. *Biochem. J.* **52**, 502–505.

Stein, E. A. & Fischer, E. H. (1958). Resistance of amylases towards proteolytic attack. *J. biol. Chem.* **232**, 867–879.

Sumner, J. B. (1925). The estimation of sugar in urine using dinitro-salicylic acid. *J. biol. Chem.* **62**, 287–290.

Taylor, R. B. (1958). Pancreatic secretion in the conscious sheep. *J. Physiol.* **143**, 81–82*P*.

Thomas, J. E. (1950). *The External Secretion of the Pancreas*. Springfield, Il.: Chas. C. Thomas.

White, T. T., Lundh, G. & Magee, D. F. (1960). Evidence for the existence of a gastro-pancreatic reflex. *Amer. J. Physiol.* **198**, 725–728.

*J. Physiol.* (1961), **158**, *pp.* 144–153
*With 5 text-figures*
*Printed in Great Britain*

# THE EFFECT OF CYCLICAL HEATING OF THE FRONT OF THE TRUNK ON THE FOREARM SWEAT RATE

By D. F. BREBNER and D. McK. KERSLAKE
*From the R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

(*Received* 10 *March* 1961)

Experiments designed to test for the presence of a neural link between thermoreceptors in the skin of one region of the body and the sweat glands elsewhere have depended on comparison of the effects of heating or cooling the first region with and without arrest of its circulation. Benzinger (1959) has dismissed such work on the grounds that a physiologically normal situation has been compared with a physiologically abnormal one, and that the stimulus used has frequently been a very cold one. Such effects as have been found (e.g. by Kuno 1956) are regarded by Benzinger as irrelevant to situations in which the sweat response is evoked by exercise or exposure to a warm environment, and he concludes that peripheral receptors are not concerned in the regulation of sweat production under normal circumstances. The present authors (Brebner & Kerslake, 1961) found that the response of the forearm sweat rate to cooling the legs was abolished by arrest of the circulation to the legs, but were unable to conclude from this that thermoreceptors in the legs were not concerned in the response, since the effect of circulatory arrest on the receptors and on the thermal stimulus to them could not be assessed.

The present experiments were designed to test for the presence of a neural link between thermoreceptors in the skin of the trunk and the sweat glands of the forearm, without the use of arterial occlusion or cold stimuli. The stimulus used was at all times warm, but varied in intensity, and the argument rests on the time relations between stimulus and response. The method also provides information about the thermal parameter to which the receptors appear to be sensitive.

## METHODS

*The heat stimulus.* Radiant heat was supplied by a bank of six reflecting lamps (Osram I.R.R. 4883, run at 115 V) arranged in two parallel rows of three each. Each lamp dissipated about 250 W. The faces of the lamps were about 1 m above the skin of the subject's trunk. 17 cm below the lamps was a grid of 14 slats, each 1·9 cm wide, separated by gaps of 1·9 cm. Around and below this grid, reflecting metal walls prevented radiation from the

lamps from reaching the subject except by passing between the gaps in the grid, and restricted the area illuminated to the region of the front of the trunk, from the clavicles to the pubis.

Immediately above this fixed grid was a second similar grid, which could be moved to and fro so as to vary the amount of radiation reaching the subject from a maximum, when the slats were superimposed, to a minimum when the slats of the movable grid overlay the gaps in the fixed grid. The movable grid was driven by a crank with a stroke of 1·9 cm, and the intensity of illumination of the skin, when measured with a photocell, was found to vary smoothly and approximately sinusoidally with time. As a result of the relatively large faces of the lamps, the proximity to them of the grids, and the distance of this system from the subject, the effect produced by movement of the grid was a variation of the intensity of illumination over the whole of the trunk surface, rather than a variation in the width of strips illuminated at constant intensity, which would have been produced had the grid been very near the skin.

The difference in radiation flux between the maximum and minimum positions of the grid was measured at the level of the subject's skin in the horizontal plane, by recording the rates of change of temperature of copper disks of various thicknesses, blackened on the upper surface and insulated on the lower. The disks were calibrated by exposure to a known radiation flux from a hot surface. Over the region occupied by the subject's trunk, the mean difference in radiation flux (the double amplitude of the cycle of radiation intensity) was 310 kcal/m$^2$ hr, with a maximum of 390 near the centre and a minimum of 200 at the shoulder.

The spectral distribution of the radiation reaching the skin should hardly be affected by the relative position of the grids, and a comparison of the emission curve for the lamps and the reflectance curve for skin (Hardy, 1949) suggests that about 20% of the radiation from the lamps would be reflected. The effective heat flux over the projected area of the trunk thus varied by about 250 kcal/m$^2$ hr between the maximum and minimum of the cycle, and the sensation produced, at the frequencies used, was one of mild but varying warmth.

*Sweat production.* Sweat was collected by passing dry air through a capsule covering 12 cm$^2$ of the volar surface of the right forearm. The effluent air was passed through an infra-red gas analyser, measuring water vapour, a sample of the dry supply air being passed at the same time through the reference tube of the analyser. The air flow was 4 l./min. The output of the analyser was arranged to control the speed of a velodyne, on the shaft of which was a perforated wheel separating a lamp and photocell. The frequency of pulses from the photocell thus depended on the sweat rate from the skin area beneath the capsule.

The pulses were fed to a set of ten counters, through a ten-way selector driven round once by each stimulus cycle. The total sweat produced in each tenth of a large number of cycles was thus recorded on the counters, the total duration relevant to each counter being recorded on a second set of counters supplied with pulses at a constant frequency. The mean sweat rate in any tenth of the recorded cycles could be found by dividing the total sweat by the total time recorded on the appropriate pair of counters.

*Calibration.* Static calibration was carried out by replacing the sweat capsule by a weighed tube from which water evaporated into the air stream. The pulse rate was found to be a linear function of the rate of evaporation over the range encountered in the experiments.

Dynamic calibration required the injection into the air stream, at the point occupied by the sweat capsule, of a water vapour cycle similar to that produced by the subjects in the experiments. As will be seen from the results, the wave form produced by the subjects was variable, and a sinusoidal change was chosen as a compromise for calibration purposes. This was produced by the system shown in Fig. 1. By adjustment of the flow in the various sections it was found possible to produce cycles of the right order of amplitude and with periods from 12 to 67 sec. Shorter periods could not be used for calibration, although used in the experi-

ments, since the performance of the system proved inadequate. The humidity cycle was driven from the crank operating the grid of the stimulator, and recording was carried out and the results analysed exactly as for a human experiment. The phase lags between the maximum of the radiation cycle and the highest recorded 'sweat rate', and between the minimum of the radiation cycle and the lowest recorded 'sweat rate' were plotted against frequency (Fig. 2). Phase lag has been expressed in tenths of a cycle rather than in degrees, to emphasize the low resolution of the system in phase. In one case the two highest readings were adjacent and equal, and this point has been plotted midway between them.

The relation between phase lag and frequency can be represented by a straight line through the origin. The time interval, $d$, between points separated by a phase angle, $\phi$, is

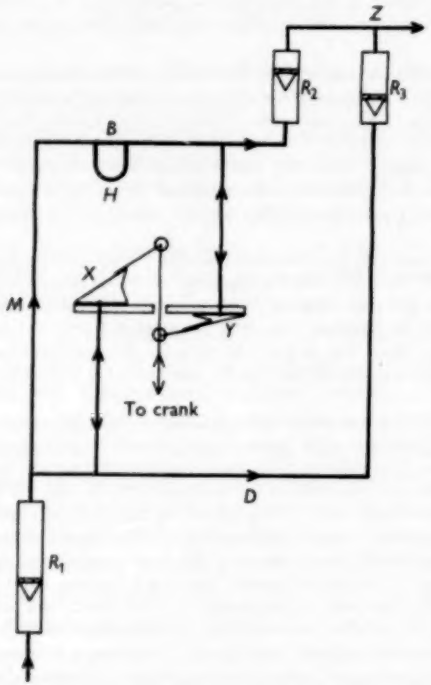



Fig. 1. Device for producing sinusoidal humidity changes for dynamic calibration of the recording system. The dry air supply, measured by the rotameter, $R_1$, is divided into two streams $M$ and $D$. Stream $M$ passes through a humidifier, $H$, with bypass, $B$. The bellows $X$ and $Y$ are driven to and fro by a crank, producing sinusoidal variations in the rate of outflow from systems $M$ and $D$ respectively. The total rate of flow is constant, since the bellows are connected in opposition, $X$ opening while $Y$ is closing and vice versa. Rotameters $R_2$ and $R_3$ are provided in order to ensure that the flows in the respective systems do not reverse. The point $Z$, where the two streams rejoin, is connected to the tube which normally leads from the sweat capsule. Humidity at this point is assumed to be maximal when the bellows are in mid position, $Y$ closing.

$\phi/f$, where $f$ is the frequency of the cycle. Since the relation between $\phi$ and $f$ is found to be linear, $d$ must be constant, and is given by the slope of the line. This means that the system behaves as if there were a constant time delay between the maximum humidity in the air leaving the capsule and the maximum counting rate of the recording system, and between the respective minima. This lag is about 7·4 sec.

*Experimental procedure.* The subject stripped and lay under the stimulator. The space between the reflecting walls and the skin and floor at the upper and lower ends was closed with blankets, so that the area exposed to the radiation was confined to the trunk from clavicles to pubis, the left upper limb and the upper half of the right arm. The right arm was abducted so that the lower half, forearm and hand were outside the radiation enclosure.

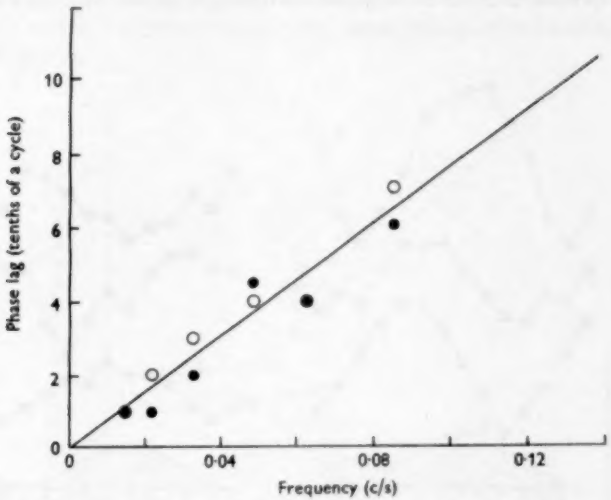



Fig. 2. Dynamic calibration of recording system; ○ maxima, ● minima. The line represents the response of a system having a constant delay of 7·4 sec at all frequencies.

These regions were further screened by blankets in order to ensure that no direct phasic heating was applied to the forearm. The room temperature was 30° C, r.h. 30 % and air movement 50 ft (12·7 m)/min. The lamps and motor of the stimulator were run for half an hour before the subject entered, so as to bring the grids up to their normal working temperature. The stimulation cycle was run continuously thereafter apart from short pauses to change gear when the frequency was changed. At such times the grid was left in the mid position.

Recordings were not started until the indicator of the gas analyser showed that sweat production had become continuous. After a stimulus frequency had been set the stimulator was allowed to run for 5 min before recording commenced. The response was then recorded for a whole number of cycles over a period as near as possible to 10 min. Stimulus frequencies were presented in random order, 3 or 4 being used at each session.

In some cases the circulation to the right arm (the same side as the sweat capsule) was arrested by inflation of a sphygmomanometer cuff to 200 mm Hg. Recording was begun 1 min after inflation of the cuff and continued for 5 min. The cuff was then deflated for 4 min and the procedure repeated.

## RESULTS

Preliminary experiments in which the output of the gas analyser was recorded continuously during cyclical heating of the trunk suggested that the sweat production was related to the stimulus. The record showed distinct peaks of sweat rate corresponding in frequency with the stimulus, but these changes were super-imposed on a very variable base line, and accurate analysis of the time relations was impossible. The rather elaborate method of recording the mean response during a series of cycles was therefore used in all subsequent work.

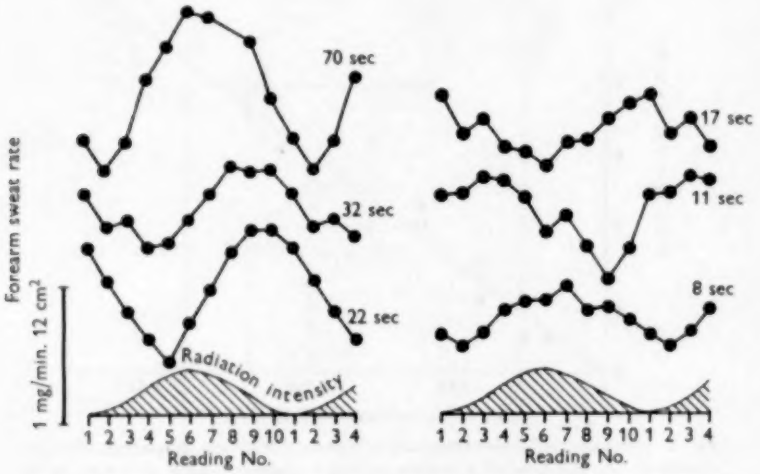

70 sec
32 sec
22 sec
17 sec
11 sec
8 sec
Forearm sweat rate
1 mg/min. 12 cm²
Radiation intensity
1 2 3 4 5 6 7 8 9 10 1 2 3 4
Reading No.
1 2 3 4 5 6 7 8 9 10 1 2 3 4
Reading No.

Fig. 3. Mean changes in forearm sweat rate during cyclical heating of the front of the trunk at various frequencies. Each curve is the mean for about 10 min continuous running. The period of the stimulating cycle is indicated for each curve. Base lines have been arbitrarily displaced in order to separate the curves.

Figure 3 shows the mean response curves for one subject at six different frequencies. Mean sweat rate is plotted against the phase angle of the stimulus, starting at minimum radiation intensity. The shapes of the response curves are somewhat irregular, but each has a reasonably well defined maximum and minimum, separated by about half a cycle.

Further analysis of the results was concerned with the time relations of the stimulus and response. From Fig. 3 it seems probable that there is an increasing phase lag as the frequency increases. At the lowest frequency (period 70 sec) the response is nearly in phase with the radiation intensity, and the same is true at the highest frequency (period 8 sec), but inspection

of the intermediate curves suggests strongly that in the latter case the response was lagging about one full cycle behind the stimulus. Since the mathematical form of the response curves was not known, no attempt was made to interpolate their true maxima and minima. Instead, the highest reading was taken as the maximum and the lowest as the minimum. This introduces a considerable grouping error—in the case of the curve for 32 sec period in Fig. 3 the maximum of a smoothly drawn curve would be almost 36° later than the highest observed reading—but since this error is also present in the calibration it is acceptable and was preferred to the arbitrary choice, for instance, of a sine wave for the representation of the response curve.

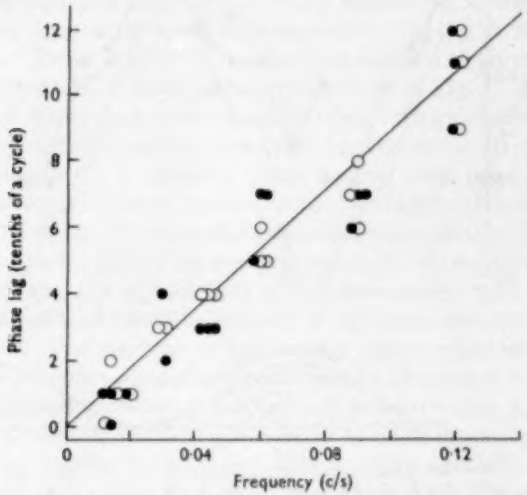



Fig. 4. Phase lags of maximum sweat rate behind maximum radiation, ○, and between respective minima, ●, plotted against frequency of the stimulating cycle. The line suggests that maximum sweat rate occurs 9·0 sec after maximum radiation intensity at all frequencies.

With radiation intensity as a reference, the phase lags of the highest and lowest recorded sweat rates in each response curve were plotted against the stimulus frequency (Fig. 4). For each subject the results may be represented by a straight line through the origin, and there is little difference between the slopes of the lines for the different subjects. A linear relation between frequency and phase lag indicates a constant time delay, which, for all results taken together, was about 9·0 sec. The intercept on the ordinate, in this case zero, indicates the fixed phase lag which may exist between the response and the reference cycle chosen. At very

low frequencies, when the 9 sec delay becomes small compared with the period of the cycle, the sweat response would be in phase with radiation intensity.

Calibration of the system with a sinusoidal humidity change showed that the delay attributable to the recording system was about 7·4 sec (see Methods). The delay between stimulus and recorded response in the human experiments was about 9·0 sec, so that the delay attributable to the human link between radiation intensity and humidity in the capsule effluent was about 1·6 sec.

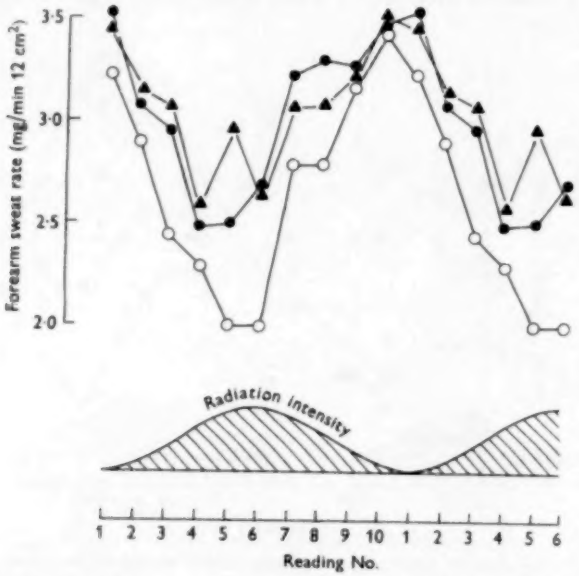



Fig. 5. Mean changes in forearm sweat rate of three subjects during cyclical heating of the front of the trunk. Period of stimulating cycle 24 sec. The circulation to the forearm was arrested for 1 min before recording commenced and maintained thereafter. Each curve is the mean for about 10 min recording.

It was thought that the stimulus would probably cause reflex changes in the cutaneous blood flow in the forearm, and that the observed changes of sweat rate might be secondary to these. In order to test this possibility experiments were performed in which the circulation to the forearm was arrested. This procedure is not open to the objections cited above (p. 144), since the receptors of the skin of the trunk would be unaffected. Provided that the response was not abolished by arterial occlusion, the conclusion could be drawn that the sweat response was not secondary to reflex changes

in the cutaneous circulation. The results are shown in Fig. 5. The magnitude and time relations of the response do not appear to be affected by arrest of the circulation to the arm.

## DISCUSSION

The forearm sweat rate has been found to follow the intensity of radiation applied to the front of the trunk with a delay of about 1·6 sec. This conclusion rests on the assumption that the method of dynamic calibration was appropriate, and is open to some objection on this ground. Dynamic calibration was performed by using a sinusoidal humidity change injected into the air stream at the point normally occupied by the sweat capsule. The choice of this wave form for calibration seems appropriate to the general form of the mean response curves (Fig. 3). Precise imitation of these wave forms would not be expected to yield delay times for the maxima and minima differing greatly from those of Fig. 2. However, the impression of the subject, which could be confirmed by observation of the indication of the gas analyser, was that in any one cycle the sweating pattern was far from sinusoidal. From one to three brief outbursts of increased sweating occurred in each cycle, the number depending on the duration of the cycle. The mean curve for a large number of cycles may thus represent the probability of an outburst occurring at any time during the cycle, rather than the typical form of the response during a single cycle.

From this point of view the method of dynamic calibration used was inappropriate. The response to brief square waves might well be a more suitable characteristic to apply. When humidity changes of this type were fed into the recording system it was found that there was an absolute delay of 4·5 sec, representing the time taken for air to reach the analyser from the capsule. After this the recorded humidity rose exponentially towards the new humidity level, with a time constant of about 6 sec. The time taken to reach a peak depended on the duration of the square wave. For waves of 1 sec duration at 10 sec intervals the delay between the start of the square wave and the peak recorded response was about 5·5 sec.

It is possible, therefore, that a more appropriate figure to use for the effective delay of the recording system is 5·5 sec. Since the total delay for the mean response curves was 9·0 sec, this increases the physiological delay to about 3·5 sec, as against 1·5 sec when the sinusoidal calibration figures are used. These two estimates probably represent the range within which the true physiological delay lies.

The observed delay of not more than 3·5 sec between stimulus and response includes the time taken for the efferent impulse to reach the sweat glands, for the glands to respond and for the secreted sweat to be evaporated into the air stream in the capsule. The remainder of the time is available

for the transmission of information from the skin of the trunk to the central end of the effector nerves. Even the time of 3·5 sec. is less than half of the lung–tongue circulation time and is too short to permit an explanation based upon the return of warm blood from the skin of the trunk. Moreover, the response was related to radiation intensity, and the skin temperature of the heated area would not reach its maximum until the radiation intensity was decaying. The results are therefore inconsistent with any mechanism which would associate maximum skin temperature with maximum stimulus. Thermal parameters which are maximal at maximum radiation intensity include the rate of change of skin temperature and the rate of heat exchange with the environment, and it seems necessary to postulate that the response depends on the stimulation of thermoreceptors in the skin of the trunk sensitive to such parameters.

The present authors (Brebner & Kerslake, 1961) found that the response of the forearm sweat rate to cooling the legs was abolished by arrest of the circulation to the legs. The results provided no evidence that the response depends on stimulation of cutaneous thermoreceptors, and it was concluded that if such receptors were involved they were not sensitive to skin temperature or to its rate of change. The possibility that they existed and were sensitive to rate of heat loss or to the temperature difference between cutaneous arterioles and venules could not be excluded. The present results are consistent with either of these possibilities, and demonstrate the existence of such receptors in the skin of the trunk.

The stimulus was strong enough to be felt by the subject, who could readily detect the times of maximum and minimum radiation intensity. However, during the experiments, the subjects frequently appeared to fall asleep and would waken later to report that they had been dreaming and had been quite unconscious of their physical environment. The sweat response did not appear to depend on the state of wakefulness of the subject and it seems unlikely that conscious sensation is involved.

## SUMMARY

1. The front of the trunk was exposed to sinusoidal variations of intensity of radiant heat, while the rate of sweat production from a sample area of the forearm was recorded. The period of the stimulation cycle was varied between 8 and 70 sec.

2. The forearm sweat rate was found to follow the radiation intensity with a delay of not more than 3·5 sec.

3. The response was unaltered by arrest of the circulation of the forearm.

4. It is considered that the findings demonstrate the existence of a

neural pathway whereby thermoreceptors in the skin of the trunk can influence the activity of the sweat glands of the forearm. The effect is not secondary to reflex circulatory changes in the forearm skin.

## REFERENCES

BENZINGER, T. H. (1959). On physical heat regulation and the sense of temperature in Man. *Proc. nat. Acad. Sci., Wash.*, **45**, 645–659.

BREBNER, D. F. & KERSLAKE, D. McK. (1961). The effect of altering the skin temperature of the legs on the forearm sweat rate. *J. Physiol.* (in the Press).

HARDY, J. D. (1949). Heat transfer. In *Physiology of Heat Regulation and the Science of Clothing*. NEWBURGH, L. H., p. 81. Philadelphia and London: Saunders.

KUNO, Y. (1956). *Human Perspiration*. pp. 218–222. Springfield: Thomas.

*J. Physiol.* (1961), **158**, *pp.* 154–164
*With 5 text-figures*
*Printed in Great Britain*

# THE MECHANICAL PROPERTIES OF THE SEMITENDINOSUS MUSCLE AT LENGTHS GREATER THAN ITS LENGTH IN THE BODY

BY J. B. DÉLÈZE*

*From the Department of Physiology, University College London*

(*Received* 10 *March* 1961)

As Blix knew in 1895, the total tetanic tension of frog's muscle passes through a maximum at a certain resting length $L'$, near to the body length $L_0$. At lengths greater than $L'$ tension decreases through a minimum, then rises again. Blix remarks that 'the relation disclosed here gives support to the idea, which has been proposed many times, that the force of contraction depends on the attraction between longitudinal tissue structures within the muscle, which are set apart by extension and in this way weaken the attraction'. The discovery of two separate sets of A and I filaments (Hanson & Huxley, 1953) and the demonstration that the A bands do not change in width during passive stretch or active shortening of muscle fibres (Huxley & Niedergerke, 1958) provided the structural basis for a revival of this type of theory of muscle contraction (e.g. Huxley, 1957). It is natural to suppose that the shear force between actin and myosin filaments, and thus the tension developed by the whole muscle, should be a function of the area of overlap of the two types of filaments; the decrease of tension development with increase in muscle length, which proved a stumbling block to many visco-elastic theories, is then readily explained. It also seems natural to suppose that other phenomena of contraction should be similar functions of the area of overlap. The purpose of this paper is to examine the mechanical properties of whole living muscles over the range of lengths beyond $L'$, where the isometric tension diminishes with increasing muscle length and to answer the following questions: (1) Does tension fall when the *active* muscle is stretched beyond $L'$? (2) Does tension rise, then fall again, if the active muscle is allowed to shorten at constant speed through $L'$? (3) Is the relation between force and speed of contraction affected beyond $L'$? And (4) is the total amount of shortening against a given load altered if contraction begins from beyond $L'$?

Before proceeding to the experiments it is necessary to discuss the

* Present address: Physiologisches Institut, Buehlplatz, Bern, Switzerland.

disagreement of some later workers with the original conclusion of Blix. Although it has never been disputed that the *extra* tension developed on stimulation passes through a maximum at a particular length and then decreases, it has been frequently reported that the total tension (resting plus active) did not pass through a minimum, but continued to rise with increasing muscle length. There is no objection to this in principle; indeed, if a muscle with very marked parallel elasticity is examined, it must show such an effect. However, this cannot be the cause of the discrepancy in the many experiments performed on frog's muscle. In some cases disagreement is explained by the use of twitches instead of tetani (Reichel, 1936; Banus & Zetlin, 1938); in others the explanation is obscure (Rosenblueth, Alanis & Rubio, 1958). Some authors have attributed Blix's minimum to the effects of fatigue (e.g. Colle, 1929); but this is certainly not the case, and Blix's results have been reproduced many times (Evans & Hill, 1914; Beck, 1922; Hill, 1925; Debler, 1936; Aubert, Roquet & Van der Elst, 1951; Abbott & Wilkie, 1953).

The division of total tension into an active and a resting part implies that these tensions are borne by elements that are separate in a mechanical, if not in an anatomical, sense; the experiments of Banus & Zetlin (1938) showed this to be at least a good approximation.

However, the experimental situation is certainly clearer when the complications due to the resting tension can be avoided over a range of lengths where tetanic tension decreases sharply. This condition cannot be achieved with the sartorius, but fortunately it can with the semitendinosus (Fig. 1). This muscle is therefore more suitable than the sartorius for the experiments that were planned.

## METHODS

British frogs (*Rana temporaria*) were used. The semitendinosus is Y-shaped, with two pelvic tendons and one tibial tendon. Each part of the muscle has fibres which are strictly parallel, but not all exactly the same length because of the mode of attachment to the tendons. The greatest variation in length is about 3 mm in a muscle of over-all length 20 mm. These experiments were all done with the anterior (ventral) part only.

The pelvic bone was clamped rigidly on a multi-electrode assembly and the tibial tendon tied to a stainless-steel wire connected to the tension or length transducers. The muscles were kept at 0° C in oxygenated Ringer's solution (mM: NaCl 115; KCl 2·0; $CaCl_2$ 1·8; Na phosphate buffer 2·0; pH 7·0).

The isotonic lever and the recording apparatus were those used by Jewell & Wilkie (1958). The mechanical performance of the light duralumin lever has been described by Jewell (1959); its equivalent mass at the point of attachment to the muscle is 0·2 g. A photoelectrical method is used for length recording and a mechano-electrical transducer tube (RCA 5734) for tension recording.

Shortening or lengthening movements at constant speeds are imposed on the muscle during a tetanus by fixing the tension transducer on the moving arm of an ergometer similar in principle to that used by Levin & Wyman (1927).

Tetani of long duration (up to 6 sec in some cases) had to be used when applying slow

length changes during contraction. It was practically impossible to keep the muscle in a perfect steady state; however, the isometric controls were fairly reproducible if long periods of recovery (at least 10 min) were allowed between the tetani. Only the experiments where controls showed no excessive decrease of tension (never more than 15 %, e.g. Fig. 2) from the beginning to the end are reported. A certain decrease of isometric tension is to be expected even under steady mechanical conditions (Aubert, 1956; Jewell & Wilkie, 1958).

## RESULTS

### *The isometric tension–length relation of two muscles*

The isometric tension–length diagrams of the frog's sartorius and of the ventral part of the semitendinosus have the same general shape (Fig. 1), but they differ in the steepness with which tension falls both above and below its maximum $P'_0$. In the case of the semitendinosus there is a long

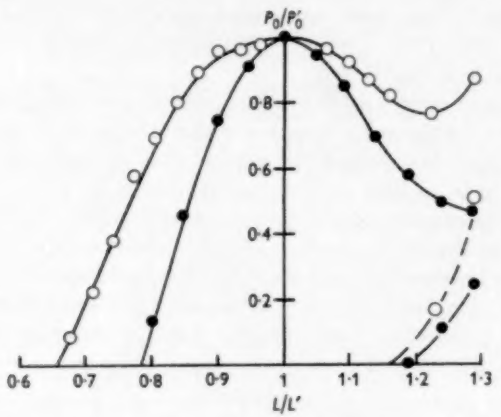



Fig. 1. Isometric tension–length curves of the sartorius ○——○ and of the semitendinosus ●——● at 0° C. The interrupted lines show the development of resting tension. To compare the shapes of the two curves, isometric tension $P_0$ is expressed as a fraction of the maximum $P'_0$, and length $L$ as a fraction of the length $L'$ at $P'_0$. $L'$ may be slightly different from the body length $L_0$. For sartorius $P'_0 = 46$ g wt., $L' = 31$ mm, $L_0 = 30$ mm; weight 57·6 mg. $P_0L_0/M = 2{\cdot}4$ kg/cm². For semitendinosus $P'_0 = 71$ g wt., $L' = 21$ mm, $L_0 = 22$ mm; weight 42 mg, $P_0L_0/M = 3{\cdot}7$ kg/cm².

region with a steep negative slope but negligible resting tension. Moreover, it is evident that the dissimilarity in the spread of the two curves does not arise merely from a difference in the resting tension; it could arise either from a different statistical distribution of $L'$ among the fibres in the two muscles, or it might reflect a regular difference in the ratio of the A to I bands in their sarcomeres.

*Effect of lengthening the muscle during tetanic contraction*

Every experiment began by an estimate of the isometric tension–length curve (see Fig. 2*A*). Length changes could then be applied over known regions of that curve.

Figure 2, *B–G*, shows records of lengthening movements at two speeds starting from three different initial lengths. The extension of 1·8 mm takes place on the positive slope of the tension–length curve (*B*, *E*), near the maximum (*C*, *F*) and on the negative slope (*D*, *G*). Each picture shows an isometric tetanus (4·5 sec) recorded at both shorter (*S*) and longer (*L*) length; the muscle is lengthened at a speed of 0·6 (*B*, *C*, *D*) or 2·8 mm/sec (*E*, *F*, *G*). The movements start 0·8 sec after the beginning of stimulation.

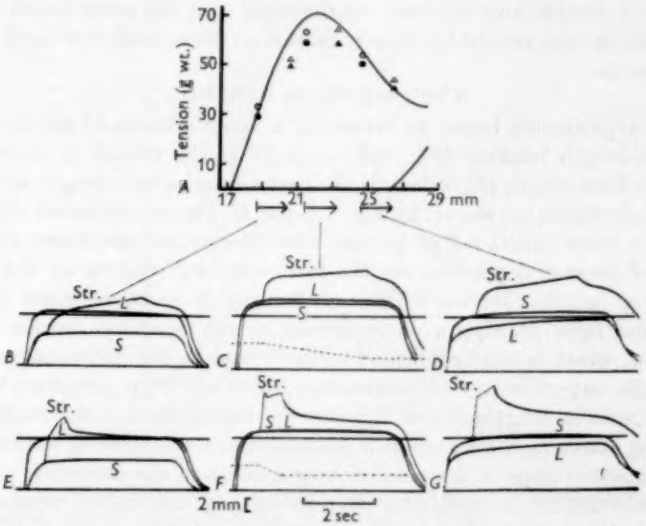



Fig. 2. Experiment to show the effect of stretching a tetanized muscle 1·8 mm in three different regions of the tension–length curve.

*A*. The continuous line shows the tension–length curve at the beginning of the experiment, and the arrows show the three regions investigated: 18·7 → 20·5 mm (records *B*, *E*); 21·4 → 23·2 mm (records *C*, *F*); 24·6 → 26·4 mm (records *D*, *G*). The symbols show the tensions actually developed after 2 sec in records *B–G*.

*B–G*. Copies of experimental records showing the tension change during a 4·5 sec tetanus at shorter length, *s*; plotted as ○ or ● in *A*.

*L*, 4·5 sec tetanus at longer length; plotted as △ or ▲ in *A*; Str., 4·5 sec tetanus with stretch; *BCD*, ○ or △, slow stretch 0·6 mm/sec; *EFG*, ● or ▲, fast stretch 2·8 mm/sec; *C*, *F* show the length change (interrupted line). The top horizontal line in each record shows the deflexion produced by 50 g wt. Semitendinosus, 0° C, 42 mg, $P_0L_0/M$ = 3, 7 kg/cm².

The tension change brought about by lengthening is complex. The most conspicuous feature is a step in tension at the start of the stretch, just as though the muscle were a viscous element. Three other features are superimposed on this step: (1) a gradual rise to a new level of tension, followed by (2) a linear increase of tension during further lengthening, more or less in proportion to the distance stretched; (3) after the end of the stretch, a very slow decay of tension. These general features of the tension change in a stretched muscle have already been fully commented on by Abbott & Aubert (1952) and by Walker (1953). The problem considered here is the way in which the effect of stretch is related to the initial length; and it is clear that tension rises more and stays high longer in *D* and *G* than in the other records. In this experiment, as in others made over a wide range of speeds and lengths, the tension *never* falls during stretch, even though the isometric tension may diminish considerably over the same length range. Thus the tension cannot be directly related to the area of overlap of A and I filaments.

*Shortening during a tetanus*

The experiments began as before by a determination of the isometric tension–length relation (Fig. 3*B*, circles). If the muscle is allowed to shorten from length (*L*) to length (*S*), tension varies with length and velocity of shortening as shown by Fig. 3*A* and *B*. The actual record (Fig. 3*A*) shows a quick initial fall of tension when the movement starts, the new level of tension depending on the force–velocity relation at the corresponding length. During further shortening at constant speed tension rises and falls, drawing a curve similar to the isometric tension–length relation, which is thus confirmed to apply also to the shortening muscle. In similar experiments on the sartorius, Aubert (1956, p. 224) finds tension to decrease at lengths below $L_0$; but the rise of tension for movements starting above $L_0$ is very small in his experiments because of the less suitable negative slope of the tension–length curve of the sartorius.

If stimulation is continued after shortening has ended, tension rises towards the isometric level and sometimes reaches it.

*Isotonic shortening during tetani starting above L'*

*Velocity.* The relations between the load and the velocity of shortening cannot be accurately investigated above *L'*, because the velocity is not constant over any appreciable distance. Shortening accelerates up to *L'*, then decelerates. Moreover, the velocity at any given length depends to some degree on the distance that the muscle has shortened already. Force–velocity curves therefore cannot be established with precision; they are roughly hyperbolic, so that Hill's equation can be tested approximately, but the values of the parameters *a* and *b*, as well as $P_0$, must be

adjusted in accordance with the length. The situation is different at lengths shorter than $L_0$, where the parameters $a$ and $b$ of the characteristic equation can be considered as independent of length (Abbott & Wilkie, 1953; Ritchie & Wilkie, 1958).

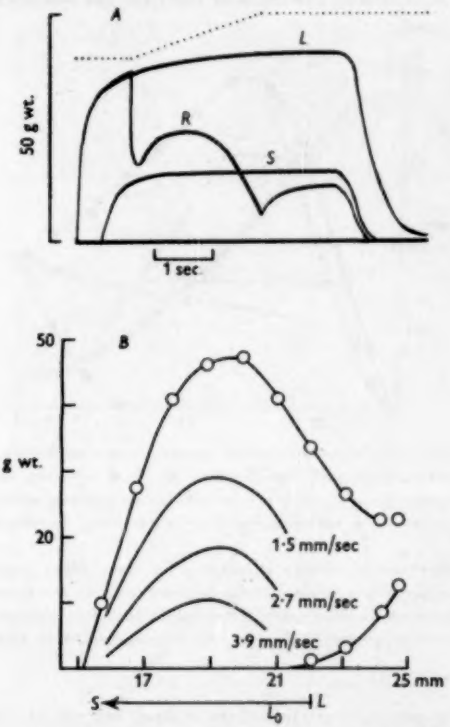



Fig. 3. *A*. Experimental record of tension change in a tetanized muscle during a release at about 2·7 mm/sec from $L'+2{\cdot}6$ mm to $L'-3{\cdot}4$ mm.

The record shows: $L$, isometric tension at $L'+2{\cdot}6$ mm; $S$, isometric tension at $L'-3{\cdot}4$ mm; $R$, tension change during a release beginning at 0·9 sec after the first stimulus. The dotted line shows the length change. Part of $R$ is plotted as the middle curve in 3*B*.

*B*. Releases at various speeds shown in relation to the tension–length curve. Only part of each record is shown. For the details consult Fig. 3*A*. Semitendinosus, 0° C; 29 mg; $P_0L_0/M = 3{\cdot}3$ kg/cm².

*Total amount of shortening.* The maximum amount of shortening varies with length as shown in Fig. 4. When shortening starts from $L'$, the same tension–length relation applies to isotonic and to isometric tetani (Abbott & Wilkie, 1953; Wilkie, 1956). The tension–length curve drawn from

isotonic *free-loaded* contractions is similar to those published by Buchthal, Kaiser & Rosenfalck (1951). If a contraction begins in the area to the left of the interrupted line, tension at the maximum of both isometric or isotonic tetanus is a function of the muscle length only, as suggested by Wilkie (1955). If it begins to the right of the line, the amount of shortening is very much reduced.

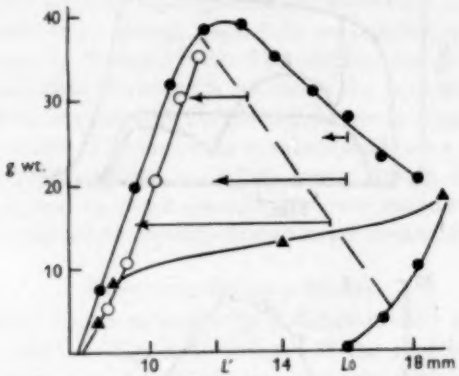



Fig. 4. Comparison of the isometric tension–length curve and of the maximum isotonic shortening from different initial lengths. ●——● Isometric tension–length curve, 1,2 sec tetani; ○——○ maximum isotonic shortening, after-loaded from $L'$, 1·2 sec tetani; ▲——▲ maximum isotonic shortening, free-loaded, 2·5–5 sec tetani.

The arrows show the maximum shortening for some other loads and initial lengths. The interrupted line is drawn from eighteen records; the isometric and isotonic tension–length curves are approximately equivalent only if length and tension remained in the area to the left of this line. Semitendinosus, 0° C, 22·3 mg, $P_oL_o/M$ = 3·4 kg/cm².

### *Effect of a previous stretch of the resting muscle on the force and velocity developed when it is stimulated*

A curious effect was observed in experiments where the resting muscle was stretched to a tension greater than about 8 g wt. Even after the muscle had been returned to an unstretched length, tension development and shortening were both slower than normal. The muscle had to spend 1 or 2 min at the shorter length before its performance recovered.

The effect on shortening is shown in Fig. 5*A*: the isotonic shortenings from $L_o$ start at different times after releasing the resting muscle from the length corresponding to 12 g of resting tension. Only after 2 min have the velocity and the total shortening recovered to their full values. Even a brief stretch has quite a marked effect, as is shown in Fig. 5*B* (compare 2 and 3); but it becomes greatest after 1 min of stretch (1).

The effect may be due to metabolic changes, for the metabolic rate of a resting muscle is increased by stretch (Feng, 1932). However, the rate of recovery is not appreciably altered by stimulating during the recovery period, though this certainly alters the metabolic rate considerably.

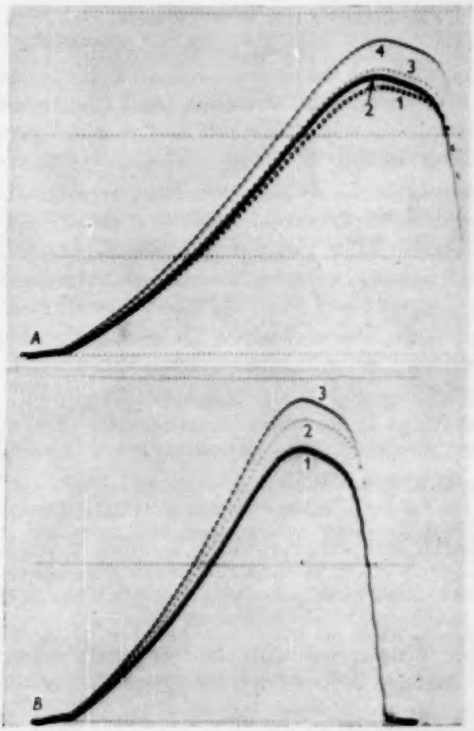



Fig. 5. *A*. Isotonic shortenings, starting from $L_{o'}$, after-load 12 g, at different times after release of the resting muscle from 12 g tension. Time after release: (1) 1 sec; (2) 10 sec; (3) 30 sec; (4) 2 min.

*B*. Isotonic shortenings, starting at $L_o$, after-load 14 g, 15 sec after release of the resting muscle from a tension of 14 g. Duration of stretch (1) 1 min; (2) 1–2 sec; (3) control after recovery. Calibration lines: mm, 50 c/s. Semitendinosus, 0° C; 25 mm; 19 mg; $P_oL_o/M = 2$ kg/cm².

## DISCUSSION

The general conclusion to be drawn from the preceding experimental results is that when an active muscle is allowed to shorten (even by only the small amount involved in registering its 'isometric' tension) the tension developed at a given speed of shortening, or the speed of shortening

against a given load, both pass through a maximum near to the length that the muscle has in the body. This length is approximately that at which the area of overlap of actin and myosin filaments is also a maximum.

However, the observed effects of stretching an active muscle are not readily explained by the sliding-filament hypothesis; for whatever the mechanism which determines the force between filaments that are being forcibly separated (see e.g. Huxley, 1957, p. 291) one would expect the force to be proportional to the area of overlap; and it is not. One possible explanation is that during such stretches, the I filaments do not actually become withdrawn from the A filaments, but that elongation and tension development occur without such displacement. At present there is no microscopic evidence to decide the question one way or the other.

Theories apart, the experimental result as it stands has some practical importance in relation to A. V. Hill's conclusion (1953) that an elongated muscle must be unstable because the parts that started strong would shorten and become stronger, while the parts that started weak would be lengthened, and would become weaker. In fact the weaker parts would be expected to resist lengthening with a considerable force that is, if anything, increased by lengthening; so instability is not inevitable.

Direct observations of muscles stimulated after elongation give somewhat contradictory results. Isolated single fibres show marked internal readjustments of length (Huxley & Peachey, 1959, 1961) while whole sartorii appear to be quite stable (Jewell & Wilkie, 1958). The difference may arise from the presence of connective tissue in the whole muscle.

## SUMMARY

1. This paper is concerned with the mechanical properties of muscle that has been extended beyond its body length. The resulting diminution in isometric tension development has often been explained in terms of a decreased area of overlap between actin and myosin filaments. If this is true, other phenomena of contraction should vary in the same way; experiments have been carried out to find out whether they do or not.

2. The experiments were performed on the frog's semitendinosus, because in this muscle the falling part of the tension–length curve is very pronounced.

3. When the tetanized muscle is lengthened at constant speed in this region, tension *never* falls but always rises; at the end of the movement tension stays above the isometric level as long as stimulation goes on.

4. When the tetanized muscle is allowed to shorten at constant speed, a series of dynamic tension–length curves similar to the isometric tension–length curve are recorded.

5. The relation between tension and length is the same for isometric and isotonic contractions within certain limits of length only.

6. Stretching the resting muscle decreases its ability to shorten or develop tension subsequently; this effect persists for some time after returning to the original length.

I am indebted to Dr D. R. Wilkie for his help and advice, and for allowing me to use the facilities of his laboratory. This work was undertaken during the tenure of a scholarship from the British Council, and finished thanks to a maintenance grant from the Swiss National Science Foundation.

REFERENCES

ABBOTT, B. C. & AUBERT, X. M. (1952). The force exerted by active striated muscle during and after change of length. *J. Physiol.* **117**, 77–86.

ABBOTT, B. C. & WILKIE, D. R. (1953). The relation between velocity of shortening and the tension–length curve of skeletal muscle. *J. Physiol.* **120**, 214–223.

AUBERT, X. M. (1956). *Le couplage énergétique de la contraction musculaire.* Thèse d'agrégation, Université de Louvain, pp. 90, 224. Brussels: Editions Arscia.

AUBERT, X. M., ROQUET, M. L. & VAN DER ELST, J. (1951). The tension–length diagram of the frog's sartorius muscle. *Arch. int. Physiol.* **59**, 239–241.

BANUS, M. G. & ZETLIN, A. M. (1938). The relation of isometric tension to length in skeletal muscle. *J. cell. comp. Physiol.* **12**, 403–420.

BECK, O. (1922). Die gesamte Kraftkurve des tetanisierten Froschgastrocnemius und ihr physiologisch ausgenutzter Anteil. *Pflüg. Arch. ges. Physiol.* **193**, 495–526.

BLIX, M. (1895). Die Länge und die Spannung des Muskels. *Skand. Arch. Physiol.* **5**, 150–206.

BUCHTHAL, F., KAISER, E. & ROSENFALCK, P. (1951). The rheology of the cross-striated muscle fibre. *Dan. Biol. Medd.* **21**, no. 7.

COLLE, J. (1929). Influence de l'excitant et du milieu extérieur sur les propriétés élastiques du muscle de grenouille. *Arch. int. Physiol.* **31**, 194–213.

DEBLER, C. (1936). Zur Dynamik des quergestreiften Muskels. *Z. Biol.* **97**, 93–98.

EVANS, C. L. & HILL, A. V. (1914). The relation of length to tension development and heat production on contraction in muscle. *J. Physiol.* **49**, 10–16.

FENG, T. P. (1932). The effect of length on the resting metabolism of muscle. *J. Physiol.* **74**, 441–454.

HANSON, J. & HUXLEY, H. E. (1953). Structural basis of the cross-striations in muscle. *Nature, Lond.*, **172**, 530–532.

HILL, A. V. (1925). Length of muscle, and the heat and tension developed in an isometric contraction. *J. Physiol.* **60**, 237–263.

HILL, A. V. (1953). The mechanics of active muscle. *Proc. Roy. Soc.* B, **141**, 104–117.

HUXLEY, A. F. (1957). Muscle structure and theories of contraction. *Progr. Biophys.* **7**, 255–318.

HUXLEY, A. F. & NIEDERGERKE, R. (1958). Measurement of the striations of isolated muscle fibres with the interference microscope. *J. Physiol.* **144**, 403–425.

HUXLEY, A. F. & PEACHEY, L. D. (1959). The maximum length for contraction in striated muscle. *J. Physiol.* **146**, 55–56*P*.

HUXLEY, A. F. & PEACHEY, L. D. (1961). The maximum length for contraction in vertebrate striated muscle. *J. Physiol.* **156**, 150–165.

JEWELL, B. R. (1959). *The mechanical properties of muscle during contraction and relaxation.* Ph.D. Thesis, University of London.

JEWELL, B. R. & WILKIE, D. R. (1958). An analysis of the mechanical components in frog's striated muscle. *J. Physiol.* **143**, 515–540.

LEVIN, A. & WYMAN, J. (1927). The viscous elastic properties of muscle. *Proc. Roy. Soc.* B, **101**, 218–243.

REICHEL, H. (1936). Quantitative Beziehungen zwischen Ruhe- und Reiz-zustand des Skelettmuskels. *Z. Biol.* **97**, 429–444.

RITCHIE, J. M. & WILKIE, D. R. (1958). The dynamics of muscular contraction. *J. Physiol.* **143**, 104–113.

ROSENBLUETH, A., ALANIS, J. & RUBIO, R. (1958). The influence of initial load and length on the amplitude of muscular contractions. *Arch. int. Physiol.* **66**, 354–372.

WALKER, S. M. (1953). Tension and excitability changes in muscle suddenly stretched during tetanus. *Amer. J. Physiol.* **172**, 37–41.

WILKIE, D. R. (1955). Living muscle. Lectures on the Scientific Basis of Medicine. **5**, 297–313.

WILKIE, D. R. (1956). The mechanical properties of muscle. *Brit. med. Bull.* **12**, 3, 177–182.

*J. Physiol.* (1961), **158**, *pp.* 165–177
*With* 11 *text-figures*
*Printed in Great Britain*



# UNIT RESPONSES FROM BRAIN-STEM NUCLEI IN THE PIGEON

BY PHYLLIS E. STOPP AND I. C. WHITFIELD

*From the Neurocommunications Research Unit, The Medical School, University of Birmingham*

(*Received* 13 *March* 1961)

Although a number of studies have been made on the microphonic (Wever & Bray, 1936; Heise & Rosenblith, 1952) and gross neural responses (Hamdi & Whitteridge, 1954; Erulkar, 1955) of the avian auditory system, no recordings appear to have been made of the response of single units in the auditory pathway of birds. Many electrophysiological studies of the mammalian auditory system at the brain-stem level have been made (for reviews see Galambos, 1954; Whitfield, 1957), and the correlation of the results so obtained with the mechanical properties imposed by the structure of the internal ear (von Békésy, 1949; Zwislocki, 1950), have led to a great number of hypotheses about the relative roles of the various sensory structures associated with the basilar membrane, and the part played by nervous mechanisms (e.g. Huggins, 1953; Allanson & Whitfield, 1955; von Békésy, 1953, 1958). Since the structure of the internal ear in birds differs considerably from that in mammals, yet the total system performs substantially the same function with the same order of acuity in the two classes, it would seem to be of great interest to compare the responses of single units in birds with their equivalents in the mammalian pathway.

The microscopic anatomy of the avian auditory pathway has been treated by Cajal (1909), by Craigie (1928), by Sanders (1929), and reviewed by Kappers, Huber & Crosby (1936). Although very little information is available about the connexions beyond the level of the brain-stem nuclei, the relation of these nuclei to the auditory nerve has been studied in several species. It seems to be generally accepted that the central ends of the fibres of the auditory division of the eighth nerve terminate upon the cells of N. angularis and N. magnocellularis of the same side. Although many of these fibres penetrate N. laminaris to reach N. magnocellularis, they are thought not to establish direct connexion with its cells. N. laminaris forms part of the auditory system, but is thought on anatomical grounds to receive its input secondarily from N. magnocellularis

(Brandis, 1894). Erulkar (1955), however, did not find any difference in the latency of the evoked auditory responses between the two nuclei.

It has been suggested that N. magnocellularis is the equivalent of the ventral cochlear nucleus in the mammal (Brandis, 1894), and it is to this nucleus that we have so far directed our attention. Preliminary results have already been reported (Stopp & Whitfield, 1960). A few records have been obtained also from N. laminaris.

## METHODS

Pigeons (which were in most cases of racing breeds, though a few examples of 'fancy' breeds were also used) were anaesthetized with urethane (1·1 g/kg) intraperitoneally. After trying a number of different substances, we found this anaesthetic to be by far the most satisfactory and to maintain a uniform depth of anaesthesia for many hours. One wing artery was cannulated to record blood pressure and the pigeon placed ventral surface uppermost. On account of the fragility of the pigeon skull and the flexibility of the bill it is difficult to clamp the head rigidly, and eventually the best method of holding it was found to be to pass a short length of stainless-steel wire through the vertex of the skull from one side to the other and to set this wire, together with the vertex, in a head holder consisting of a shallow trough about one quarter inch deep containing plaster of Paris. In this way the skull was held firmly without impeding the auditory, visual or respiratory pathways. The brain stem was exposed from the ventral aspect by removal of the trachea and the musculature in the triangle formed by the separation of the carotid arteries, and by nibbling away with forceps the bone so exposed. This bone was removed as far caudally as a point just caudal to the tip of the odontoid process and for a distance of about 2 mm rostral to it. Removal of the bone further rostral than this is liable to damage the cochleae. After this dissection was completed, the pigeon head holder was clamped in a stereotaxic apparatus so that the line of the bird's beak cleft was parallel to the horizontal reference plane of the instrument.

Stimuli were led to the two ears through short Perspex tubes, terminated by ear-moulds which had been cast to fit the external ear. The stimuli were generated in most cases by Brush crystal BA-206 earpieces fed from two audio oscillators via a mixer, an audio amplifier, and suitable compensating networks. The over-all response was in this way made reasonably uniform over the range 250–6000 c/s. In some experiments, and for lower frequencies, S.G. Brown 'Super K' moving coil earpieces were employed.

Glass micropipettes of about 0·5 $\mu$ outer tip diameter filled with 5 M-NaCl and mounted on a Grass P-6 probe were used for recording. They were inserted into the brain stem under direct vision, using a Zeiss otoscope. One eyepiece of the otoscope contained a graticule which superimposed a grid on the image of the brain stem. Orientation of this grid on surface markings facilitated successive insertions of the electrode in known positions. In order to reach the nucleus, the electrode carrier was set to drive the electrode in a ventro-rostral–dorso-caudal direction making an angle of about 30° with the horizontal plane of the instrument.

At the end of each successful experiment the micropipette was left at the recording site and the brain perfused with saline and formaldehyde solution via the cannulated wing artery. The fixed brain was then sectioned in the plane of the electrode and stained with Luxol fast blue and Cresyl violet (Klüver & Barrera, 1953).

RESULTS

From 68 pigeons which survived anaesthesia and operative procedures, a total of 100 auditory units were isolated and studied. Examples of the responses of such units to stimulation are shown in Fig. 1. All units which were excitable by tonal stimulation showed the typical threshold responses which have been described in mammals (Galambos & Davis, 1943; Tasaki & Davis, 1955); that is to say, they had well-defined characteristic frequencies at which the threshold was lowest and a progressively rising

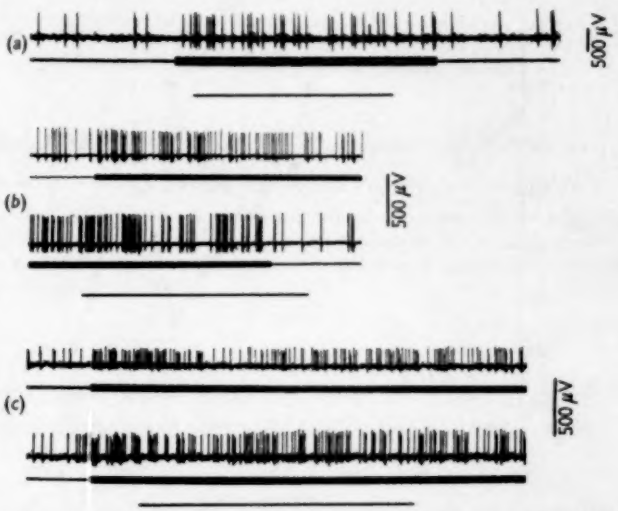



Fig. 1. Responses of single units in nucleus magnocellularis to acoustic stimulation at their characteristic frequencies: (*a*) 1450 c/s, −35 db. (*b*) 2600 c/s, −10 db; upper trace, onset of stimulation; lower trace, termination of stimulation. (*c*) 1950 c/s, −30 db (upper trace) and −10 db (lower trace).

Time bar (below each set of records) 0·5 sec. The sound reference level in this and subsequent figures is approximately 1 dyne/cm²; and stimulation is indicated by thickening of the signal line immediately below each trace.

threshold for frequencies further removed from this value. The great majority showed the 'classical' type of curve rising more steeply on the high frequency side (Fig. 2), but nevertheless a few anomalous curves of the type described by Katsuki, Watanabe & Suga (1959) were also seen. These curves were more nearly symmetrical or even tended to be mirror-images of the classical curves, with the low-frequency side rising the more abruptly (Fig. 2, full line). The absolute values of the thresholds varied considerably from unit to unit, as they have been found to do in cats, and

in fact covered about the same range as those observed in mammalian studies.

In 'low-frequency' units the same definite relationship has been seen between the response and the phase of the stimulating sinusoid (Fig. 3*a*), as is commonly observed in cats. However, this was not always the case, and Fig. 3*b* illustrates a unit where the relative phases show considerable variability. This failure to 'lock' was not related to stimulus frequency and occurred whether the stimulating tone was near to (Fig. 4*b*) or distant from (Fig. 4*a*, *c*) the characteristic frequency.

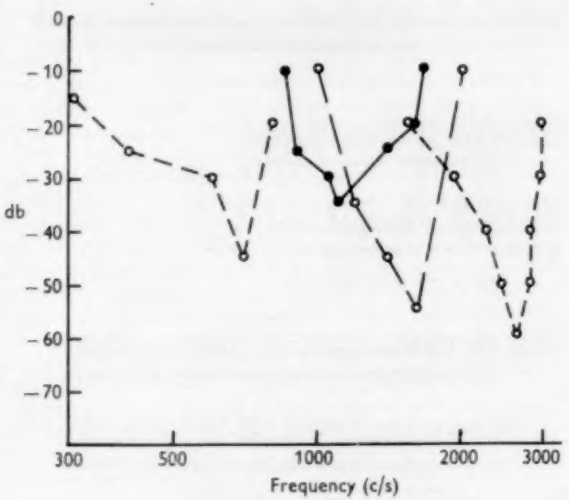



Fig. 2. Typical threshold–frequency response curves for four units. The majority of such curves follow the 'classical' pattern but some 'anomalous' curves (full line), which are symmetrical or rise more abruptly on the low-frequency side, are found.

A proportion of the units studied (twenty-one) could not be excited by any stimulus which was available to us, but their resting activity could nevertheless be readily inhibited by tonal stimuli (Fig. 5), the frequencies adequate for this being well defined and showing similar behaviour to other inhibiting stimuli (see below).

In almost every case the 'spontaneous' firing rate was higher than that found in the trapezoid body in cats (Hilali & Whitfield, 1953). Although spontaneous rates as low as 10/sec were seen, rates as high as 100/sec were commonly observed and these might vary by as much as 60 % from minute to minute in the same unit. With the termination of the stimulating tone

the spontaneous activity was temporarily depressed (Fig. 6); almost all units studied displayed this 'silent period'. Stimuli of equal magnitude produced increases of anything from 5 to 195% above the spontaneous firing rate in different units.

Units with characteristic frequencies ranging from 90 to 4130 c/s have been isolated, the most numerous being those responding around 1800 c/s. The distribution of characteristic frequencies of the units studied is shown in the form of a histogram in Fig. 7. It is probable that units responding

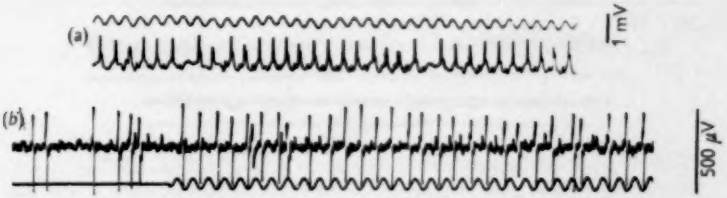



Fig. 3. The relation between neural discharge and phase of the stimulating sinusoid: (*a*) Constant relationship in a unit stimulated at its characteristic frequency of 285 c/s. (*b*) Variable relationship in a unit of characteristic frequency 450 c/s, stimulated at 250 c/s.

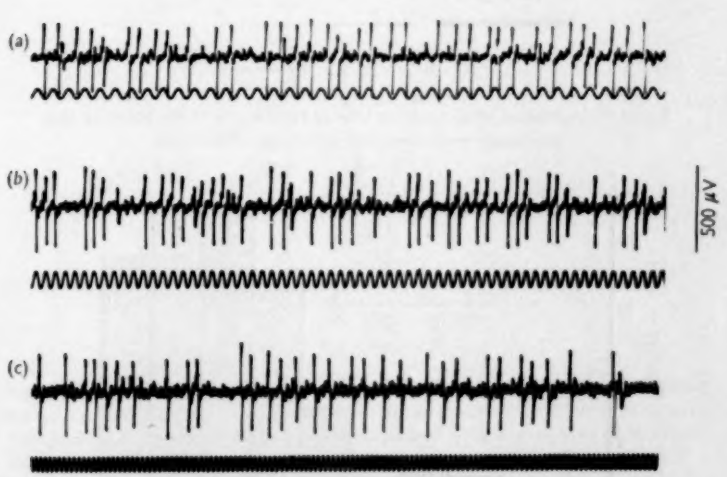



Fig. 4. Same unit as Fig. 3*b*, showing responses to activating frequencies which are lower than, close to, and higher than the characteristic frequency of 450 c/s. (*a*) 250 c/s −10 db; (*b*) 400 c/s −25 db; (*c*) 800 c/s −10 db. The relationship with the stimulating sinusoid is irregular at all frequencies. In the case of record (*c*) it was necessary to enlarge successive portions of the record to verify this.

to frequencies below 90 c/s exist, but our equipment was not adequate for making quantitative observations in this region.

In order to determine the relative threshold of a given unit to stimulation from the homolateral and contralateral sides, tones were applied separately to each ear. In no case was a lower threshold found for stimulation from the contralateral side, the contralateral thresholds being in general 25–40 db higher than those on the same side as the nucleus.

Fig. 5. (*a*) Partial inhibition of a unit which could not be excited; inhibiting tone 1800 c/s, − 10 db. (*b*) Complete inhibition at − 55 db of another 'inexcitable' unit; inhibiting tone 250 c/s.

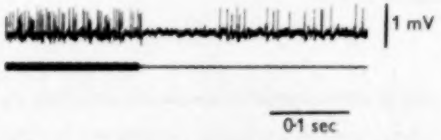



Fig. 6. Termination of an exciting tone of 1900 c/s, − 10 db, showing the temporary suppression of spontaneous activity.

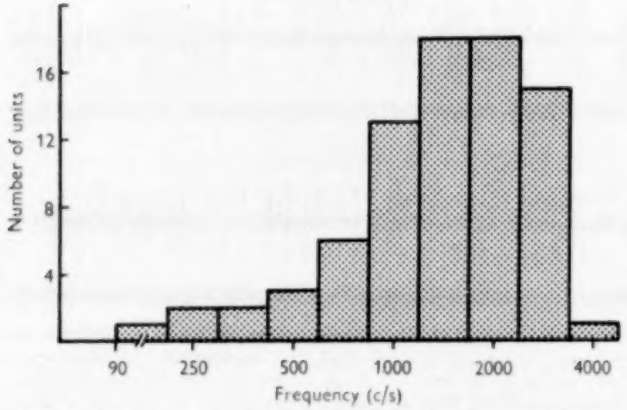



Fig. 7. Distribution of characteristic frequencies of units studied.

Of the units studied approximately two-thirds could be inhibited by tonal stimuli, while the remainder could not be so inhibited by tones within the range tried (100–10,000 c/s). Although some units required inhibitory tones of the same range of intensity as the exciting tone

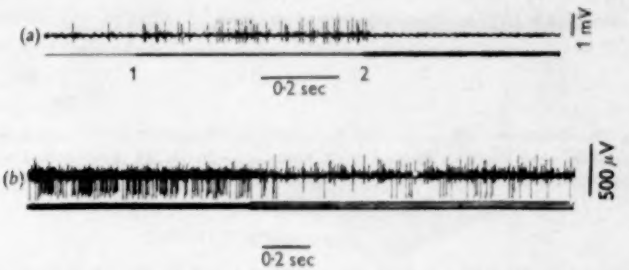



Fig. 8. (*a*) Unit excited (1) at its characteristic frequency of 280 c/s, −20 db. The addition of a second tone (2) of 570 c/s at the same intensity inhibited all activity. (*b*) Unit excited at its characteristic frequency of 1600 c/s. Addition of a second tone of 885 c/s produced only partial inhibition even though the intensity of this inhibiting tone was 48 db stronger.

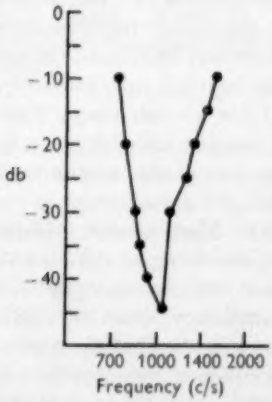



Fig. 9. An inhibitory response curve showing the frequency–intensity relationship for which the inhibiting tone just produces a measurable diminution in firing rate. The general form of this curve may be compared with the excitatory threshold response curve of Fig. 2.

(Fig. 8*a*), others required inhibitory tones of considerably greater intensity to produce a significant effect (Fig. 8*b*). Even with quite strong stimuli inhibition was in some cases only partial. An inhibitory response curve is shown in Fig. 9.

In the course of the experiments, a few records (8 units) were obtained from sites in N. laminaris (Fig. 10). These units appeared to respond in a manner indistinguishable from units in N. magnocellularis, judged on the small sample available.

Fig. 10. Responses of a single unit in nucleus laminaris. At (*a*) it is excited by a tone of its characteristic frequency of 2700 c/s, −60 db. At (*b*) a second tone of 4500 c/s, −10 db is added which only partially inhibits activity.

## DISCUSSION

The range of hearing of pigeons has been investigated by subjective methods at various times since the early experiments of Wundt (1894). Using training methods, Jellinek (1926) reported positive results for discriminations with tones between 652 and 3480 c/s in turtle doves. Wassiljew (1933), using conditioning methods in pigeons, placed the upper limit in the region 10,000–12,000 c/s. Huizinga (1935), using the sound reactions of Tullio (1929), found that the effective range for these responses was 650–8277 c/s. These authors attributed the reactions to stimulation of the vestibular part of the internal ear, however, since extirpation of the cochlea reduced but did not abolish them. They were only seen in the presence of an artificial opening into the bony labyrinth. Wever & Bray (1936) obtained cochlear microphonic responses from the pigeon over the range 100–10,000 c/s, though the magnitude of the response started to fall sharply above 3–4000 c/s. More recently Heise (1953), using operant conditioning techniques, has obtained detailed threshold response curves for the pigeon. He found the threshold to be lowest in the region of 2000 c/s and to rise abruptly above 4000 c/s. The low-frequency part of the curve was stated to be similar to that for man.

The upper limit of frequency response in our experiments coincides fairly exactly with Heise's results, the highest characteristic frequency we have found being 4130 c/s. As is shown in Fig. 6, units with characteristic frequencies around 1800 c/s are the most common. Although we have not been able to determine threshold–frequency relationships for units with responses below 100 c/s owing to the limitations of our equipment, we have evidence of the existence of such responses—possibly down to frequencies as low as 40 c/s.

The structure of the avian auditory receptor is very different from that in mammals (Fig. 11). Estimates of the number of hair cells vary from

1200 to 3000 (Portmann, 1950; Prosser, Bishop, Brown, Jahn & Wulff, 1950), a finding which might be correlated, at least in the pigeon, with the shorter range of hearing. However, the most striking difference is in the arrangement of the cells. There is nothing corresponding to the mammalian organ of Corti, and hence no division into inner and outer hair cells. Furthermore, the number of these undifferentiated cells forming one transverse row is much greater than in the mammalian organ.

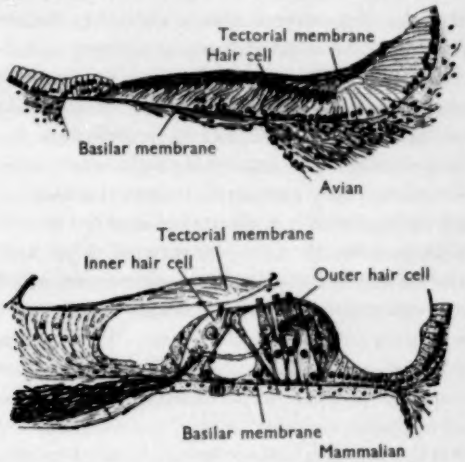



Fig. 11. Comparative structure of avian and mammalian auditory receptor organs. Upper figure after Satoh (1917); lower figure after Retzius (from Cajal, 1909).

It would be expected that the mechanical behaviour of the two types of structure would be somewhat different, and von Békésy (1944) has shown that the mechanical resolving power of the bird basilar membrane (in terms of the distance the point of maximum displacement is shifted for a given frequency change) is lower in birds than in any of the mammals. Yet we find that the threshold–response curves at the level of nucleus magnocellularis are virtually identical with those found in the cat cochlear nucleus. Such findings would support the view (Whitfield, 1956, 1959) that provided there is *some* differential response at the periphery, it is the nervous portion of the auditory system which plays the major part in extracting this difference and transforming it into a form utilizable by the higher levels of the nervous system. Our findings suggest that this process may have already gone so far at the brain-stem level that a similar informational pattern is presented to the higher levels of the nervous system in both birds and mammals.

In such a process it has often been suggested (e.g. Huggins, 1953) that comparison of the relative responses of the inner and outer hair cells by the nervous system plays an important role in the transformation. However, if this is so the role cannot be a very critical one, since no differentiation into inner and outer hair cells exists in the pigeon. It is true, of course, that a comparison of the outputs of cells in different positions across the row might perform a similar function, but this would not give the clear dichotomy in behaviour which has so far been demanded by such hypotheses. In the absence of positive evidence about the function of inner and outer hair cells the question must still remain a very open one.

It would likewise seem that the difference between the symmetrical threshold–response curves of Katsuki (Katsuki *et al.* 1959) and the usual asymmetric type cannot be attributed to a difference in origin of their afferent excitation in outer and inner hair cells, since both these types of curve have been seen in the pigeon as well as in the cat.

Although some examples of low spontaneous activity were observed, the rates of spontaneous activity were, in general, high and the increases produced by quite strong stimulation were sometimes only small. Furthermore, the rates of spontaneous activity would commonly fluctuate quite widely in the same unit over a period of time. We have therefore found it difficult to draw any conclusions about the relation between stimulus strength and response in these experiments. Whether such high resting rates are normally present, or whether they are an artifact produced by the introduction of the recording electrode, we cannot at present say. Certainly the cells of N. magnocellularis are very closely packed and a close approach is necessary to isolate the response of one of them. High rates of spontaneous firing are commonly observed in the mammalian cochlear nucleus also, and it seems at least possible that we ought not to rely on data about firing rates recorded from the interior of nuclei, but consider, for this purpose, only axonal responses.

The observation that thresholds were some 25–40 db higher on the contralateral than on the homolateral side is in agreement with the anatomical evidence that the auditory nerve terminates entirely in the nuclei of the same side. Although the results have not been confirmed by cochlear destruction, it would appear reasonable to attribute a difference of this order to mechanical cross-transmission. The attenuation over such a non-nervous pathway might be expected to result in a threshold difference of about the magnitude found.

Galambos & Davis (1944) found inhibitory phenomena present at a quite peripheral position in the cat cochlear nucleus; Tasaki & Davis (1955) found evidence in the guinea-pig for two distinct groups of fibres coming from the periphery (as judged on a basis of threshold) which they suggested

might represent those taking origin from inner and outer hair cells respectively. Allanson & Whitfield (1955) examined the implications of inhibitory afferent fibres arising at the periphery, but rejected this hypothesis on grounds of its rather precise threshold demands, in favour of inhibitory connexions within the nucleus itself. However, as these authors stated, the evidence for rejecting peripheral inhibitory fibres was not strong. In view of the foregoing, the inhibitory phenomena in the pigeon are of particular interest.

In general, these inhibitory phenomena seem to parallel those in mammals. The inhibiting frequencies are specific, there being for each unit a particular value which is the most effective. The relation between the intensity of the inhibiting tone and the band of frequencies over which it influences the response follows the same general triangular pattern seen in cats (Fig. 9), and is obviously analogous to the excitatory threshold curves.

As in the mammalian system, there are many units which apparently cannot be inhibited by tonal stimuli. A striking observation lay in the number of units (20%) in which the spontaneous activity could be inhibited by specific frequencies, but which could not be excited by any stimulus we had available. As far as we are aware this is not paralleled in mammalian studies.

## SUMMARY

1. The responses to sound stimulation of 100 units from auditory nuclei in the brain stems of pigeons, mostly from N. magnocellularis, have been examined.

2. The threshold–response curves obtained were essentially similar to those which have been found in mammals.

3. The range of frequencies to which responses were obtained extended from below 100 c/s to just over 4000 c/s.

4. Two thirds of the units studied could be inhibited by tonal stimuli. About one fifth of all units examined could not be excited under the conditions used, but their resting activity could be inhibited.

5. At low frequencies the responses were usually, but not invariably, related to the phase of the stimulating sinusoid.

6. The threshold of all units studied was consistently much lower for stimulation of the homolateral than of the contralateral ear.

7. Observed rates of resting discharge were in general high.

8. In spite of the considerable dissimilarity in the structure of the mammalian and avian internal ears, no striking differences, which could be attributed to this source, have been seen in the relative behaviour of the neural units.

We should like to thank the Medical Research Council for a grant of materials and for a maintenance grant to one of us (P.E.S.), and the Wellcome Foundation for a travel grant. Our thanks are also due to Dr J. J. Elkes and the United States National Institute of Mental Health, under whose auspices the preliminary part of this work was done, for the loan of a number of pieces of equipment. The assistance of Dr George Paulson in the preliminary work of determining the stereotaxic coordinates of the nuclei is gratefully acknowledged.

## REFERENCES

ALLANSON, J. T. & WHITFIELD, I. C. (1955). The cochlear nucleus and its relation to theories of hearing. *Third London Symposium on Information Theory*, pp. 269–286. London: Butterworths.

BRANDIS, F. (1894). Untersuchungen über das Gehirn der Vögel. II Theil: Ursprung der Nerven der Medulla oblongata. *Arch. mikr. Anat.* Bd. **43**, 96–116.

CAJAL, S. R. (1909). *Histologie du système nerveux de l'homme et des vertébrés*. Paris: Maloine.

CRAIGIE, E. H. (1928). Observations on the brain of humming bird (*Chrysolampis mosquitus* Linn. and *Chlorostilbon caribaeus* Lawr.). *J. comp. Neurol.* **45**, 377–482.

ERULKAR, S. D. (1955). Tactile and auditory areas in the brain of the pigeon. An experimental study by means of evoked potentials. *J. comp. Neurol.* **103**, 421–458.

GALAMBOS, R. (1954). Neural mechanisms of audition. *Physiol. Rev.* **34**, 497–528.

GALAMBOS, R. & DAVIS, H. (1943). The response of single auditory nerve fibres to acoustic stimulation. *J. Neurophysiol.* **6**, 39–58.

GALAMBOS, R. & DAVIS, H. (1944). Inhibition of activity in single auditory nerve fibres by acoustic stimulation. *J. Neurophysiol.* **7**, 287–303.

HAMDI, F. A. & WHITTERIDGE, D. (1954). The representation of the retina on the optic tectum of the pigeon. *Quart. J. exp. Physiol.* **39**, 111–119.

HEISE, G. A. (1953). Auditory thresholds in the pigeon. *Amer. J. Psychol.* **66**, 1–19.

HEISE, G. A. & ROSENBLITH, W. (1952). Electrical responses to acoustic stimuli recorded at the round window of the pigeon. *J. comp. physiol. Psychol.* **45**, 401–412.

HILALI, S. & WHITFIELD, I. C. (1953). Responses of the trapezoid body to acoustic stimulation with pure tones. *J. Physiol.* **122**, 158–171.

HUGGINS, W. H. (1953). *A Theory of Hearing*. Air Force Cambridge Research Center Technical Report 14–53, Cambridge, Mass., U.S.A.

HUIZINGA, E. (1935). On sound reactions of Tullio. *Acta otolaryng., Stockh.*, **22**, 359–369.

JELLINEK, A. (1926). Versuche über das Gehör der Vögel. *Pflüg. Arch. ges. Physiol.* **211**, 64–72.

KAPPERS, A., HUBER, G. C. & CROSBY, E. C. (1936). *The Comparative Anatomy of the Nervous System of Vertebrates, including Man*. Vol. I, pp. 473–484. New York: The Macmillan Co.

KATSUKI, Y., WATANABE, T. & SUGA, N. (1959). Interaction of auditory neurons in response to two sound stimuli in cat. *J. Neurophysiol.* **22**, 603–623.

KLÜVER, H. & BARRERA, E. (1953). A method for the combined staining of cells and fibres in the nervous system. *J. Neuropath.* **12**, 400.

PORTMANN, A. (1950). *Traité de zoologie*, P. P. Grassé. Tome XV: Oiseaux, p. 205. Paris: Masson and Co.

PROSSER, C. L., BISHOP, D. W., BROWN, F. A., JAHN, T. L. & WULFF, V. L. (1950). *Comparative Animal Physiology*. Philadelphia: W. B. Saunders Co.

SANDERS, E. B. (1929). A consideration of certain bulbar, mid-brain and cerebellar centres and fibre tracts in birds. *J. comp. Neurol.* **49**, 155–222.

SATOH, N. (1917). Der histologische Bau der Vögelschnecke. Basel: Schwabe.

STOPP, P. E. & WHITFIELD, I. C. (1960). Auditory responses from the brain stem of pigeon. *J. Physiol.* **151**, 21–22*P*.

TASAKI, I. & DAVIS, H. (1955). Electric responses of individual nerve elements in cochlear nucleus to sound stimulation (guinea-pig). *J. Neurophysiol.* **18**, 151–158.

TULLIO, P. (1929). Some experiments and considerations on experimental otology and phonetics. Bologna: Cappelli.

VON BÉKÉSY, G. (1944). Über die mechanische Frequenz-analyse in der Schnecke verschiedener Tiere. *Akust. Z.* **9**, 3–11.

VON BÉKÉSY, G. (1949). The vibration of the cochlear partition in anatomical preparations and in models of the inner ear. *J. acoust. Soc. Amer.* **21**, 233–245.

VON BÉKÉSY, G. (1953). Shearing microphonics produced by vibration near the inner and outer hair cells. *J. acoust. Soc. Amer.* **25**, 786–790.

VON BÉKÉSY, G. (1958). Funneling in the nervous system and its role in loudness and sensation intensity on the skin. *J. acoust. Soc. Amer.* **30**, 399–412.

WASSILJEW, M. P. (1933). Über das Unterscheidungsvermögen der Vögel für die hohen Töne. *Z. vergl. Physiol.* **19**, 424–438.

WEVER, E. G. & BRAY, C. W. (1936). Hearing in the pigeon as studied by the electrical responses of the inner ear. *J. comp. Psychol.* **22**, 353–363.

WHITFIELD, I. C. (1956). The sensory pathway: the nervous mechanism of hearing. *Advanc. Sci., Lond.*, **12**, 439–442.

WHITFIELD, I. C. (1957). The physiology of hearing. In *Progr. Biophys.* **8**, 1–47.

WHITFIELD, I. C. (1959). Sensory mechanisms and sensation. *N.P.L. Symposium on 'The Mechanisation of Thought Processes'*, pp. 357–368. London: H.M. Stationery Office.

WUNDT, W. (1894). Akustische Versuche an einer labyrinthloser Taube. *Phil. Stud.* **9**, 496–509.

ZWISLOCKI, J. (1950). Theory of the acoustical action of the cochlea. *J. acoust. Soc. Amer.* **22**, 778–784.

*J. Physiol.* (1961), **158**, *pp.* 178–196
*With 4 text-figures*
*Printed in Great Britain*

# THE NEGATIVE DELAYED HEAT PRODUCTION IN STIMULATED MUSCLE

By A. V. HILL
*From the Department of Physiology, University College London*

With an appendix by R. C. WOLEDGE

(*Received* 21 *April* 1961)

In a paper on the delayed ('recovery') heat production of frog sartorius muscles, Hartree (1932*a*) described experiments at 0° C in nitrogen which appeared to show an absorption of heat lasting for about 40 sec after tetanic contractions of 1–5 sec duration. This 'negative' heat production amounted, on the average, to 3 % of the initial heat, varying from 1 to 5 %. Six years earlier Furusawa & Hartree (1926), working with frog muscles in nitrogen at room temperature, had reported the frequent observation of early negative delayed heat after a short stimulus (0·05 sec or less); it amounted to 2–4 % of the initial heat. After a longer stimulus (0·3 sec) it was absent. They were inclined to regard it as an error due to irregular distribution of the heat production, though they had taken precautions to avoid such effects by using a special thermopile making contact with both faces of the muscle. In view of the results of the present paper it seems likely that the effect they observed was genuine: but their doubt about its reality is shown by the fact that they did not mention it in the summary of their paper. Two years later Hartree & Hill (1928, p. 210) reported 'in our experience there is never any negative heat'. Its possibility was in their minds, because Nachmansohn (1928) had just discovered that up to 30 % of the phosphagen split during a tetanus was resynthesized in about 20 sec at room temperature in the absence of oxygen: and he claimed that no lactic acid was formed at the same time, a result shown 3 years later, by the work of Lundsgaard (1931) and of Lehnartz (1931), to be wrong. Had his result about lactic acid been correct there might have been a large negative heat: though it is not evident then where the free energy could have come from to drive the resynthesis of the phosphagen.

Because of the special interest of his result, and because of previous doubts and failures with negative heat, Hartree examined the conditions of his experiments very critically. The negative heat was small and could possibly have been due to several errors that might have affected his measurements. He was able to discard any possible errors which could be

attributed to irregularities, if they occurred, in the distribution of heat within the muscles; the time course of the negative heat was much too long to be explained in this way. He was inclined to believe that it might be due to an error in his 'heating control' curves; this could be caused by a small change of weight of the muscle on the thermopile between the times (1) when the records of heat production were made on the living muscle and (2) when those of artificial electrical heating were made on the dead muscle after it had been killed by chloroform vapour. If the weight had been increased (e.g. by the absorption of water vapour due to increased osmotic pressure, or by slight shortening and thickening) the muscles would cool more slowly after electrical heating: then the analysis of the heat production in the living muscle would show an apparent absorption of heat.

Hartree was the more inclined to accept this explanation of the effect observed, because he found it to occur equally in muscles adequately poisoned with iodoacetate, in which lactic acid is not produced. This seemed to eliminate the possibility that the apparent negative heat could be due to the restoration of creatine phosphate by means of the free energy of lactic acid formation. He was not really satisfied, however, with dismissing, as a technical error, an effect he had observed so regularly and he returned to the subject in a later paper (Hartree, 1932*b*), employing a new method of making his heating control curves. Instead of killing the muscles with chloroform and using an alternating current of low frequency for heating, he adopted a valve oscillator providing an alternating current of frequency high enough (100 kc/s) not to stimulate. With this the heating control could be made on the living muscles at any time during a series of records of the heat production due to stimulation. His previous observations were entirely confirmed. In nitrogen at 0° C, after a 1 sec stimulus, there was negative heat production completed in 15–50 sec and varying from 1 to 8 % of the initial heat. After a long stimulus (e.g. of 10 sec) negative heat was not found: it was masked, Hartree surmised, by the onset of the usual positive delayed heat.

Two years later Bugnard (1934), using an extremely accurate method with single twitches at room temperature of a muscle in nitrogen, obtained clear evidence of negative delayed heat. It amounted to about 4 % of the initial heat and appeared in the first 15–20 sec after the shock. In oxygen also Bugnard found signs, in the first 10–15 sec, of negative delayed heat. Both in oxygen and in nitrogen the negative heat was followed by the usual positive heat, which presumably cut it short. After single twitches the positive delayed heat, whether aerobic or anaerobic, comes on very slowly compared with that after even a short tetanus. That probably is why Bugnard was able to detect the negative heat at room temperature.

The only other published reference to negative delayed heat is that by

D. K. Hill (1940). But his observations were only incidental in an investigation of the time course of the oxygen consumption, of the recovery heat and of the pH changes in muscles stimulated at 0° C. His results added nothing material to Hartree's.

The importance of Hartree's and Bugnard's results, if confirmed, in connexion with the thermodynamics of the chemical processes known, or supposed, to follow contraction made it desirable to examine the negative heat again; and in particular to find out whether this negative heat was uniquely characteristic of 0° C and lack of oxygen, or whether it occurred also, as Bugnard's results suggested, in the presence of $O_2$ and at higher temperatures. Greater precision is possible today than with the thermopiles available to Hartree in 1932, particularly because of the valuable property of the present ones that the rate of heat loss of muscles lying on them is exponential: which means that the course of the delayed heat production can be calculated from the records, over long times, with greater accuracy and far less labour than earlier.

The new examination of the negative delayed heat not only has confirmed its existence at 0° C in the absence of oxygen, and the general description of it by Hartree, but has shown (1) that it occurs also at room temperature (15–20° C), and (2) that it is apparent at both temperatures in oxygen as well as in nitrogen; though in oxygen it is cut short and partly masked by the usual recovery heat. It is a general phenomenon, therefore, and must be taken into account in any discussion of the chemical events that occur after contraction and relaxation.

## METHODS

*Electrical heating controls.* Measurement of the delayed heat production of muscle always requires the use of a 'control' curve made by electrical heating of the muscles lying on the thermopile exactly as they were for the records of the heat production due to stimulation. The only purpose today of these heating control curves is to provide the constant needed for an accurate allowance for heat loss: the rest of the analysis (i.e. the allowance for time lag in thermopile and galvanometer) is carried out by other means (Hill, 1949*b*). The fall of temperature, after the initial heat production, is rapid, 2·5 %/sec in the larger muscles used, 5 % in the smaller ones. The former would reduce the initial rise of temperature within 1 min to 22 %, the latter to 4·6 %; and there is no exact method of allowing for such heat loss except by making a heating control curve in every experiment. Before 1938 the allowance was very laborious, involving successive subtractions, as in the older form of analysis (see Hartree, 1931, Appendix II), over the whole period (minutes) in which the delayed heat appeared.

With most of the thermopiles constructed since 1938, however, this labour is avoided by the fact that the fall of temperature of the muscles on them is accurately exponential (Hill, 1939). The rate of heat loss is embodied in a single time constant, derived from the heating control, which can be applied to any heat record from start to finish. When the records of the heat produced by the stimulated muscles have been measured and tabulated, the rest of the operation, using this time constant, can be carried out with an adding machine. The final result is a set of numbers from which the true curve of heat production can be plotted.

In order to ensure that the fall of temperature due to heat loss is accurately exponential the following precautions are needed:

(1) The behaviour of the thermopile must be carefully examined: if the fall of temperature is not accurately enough exponential the thermopile should not be used for measuring delayed heat.

(2) The part of the muscle lying on the thermopile should be as uniform as possible; if the cross-section varied, the heating current would produce differences of temperature along the muscles.

(3) Special heating electrodes should be provided, lying between the muscles in the plane of the thermopile, several millimetres beyond its ends. The reasons for this are (*a*) that at the heating electrodes themselves the current lines are concentrated, so the rise of temperature there is greater than in the middle region of constant current density; and (*b*) in the regions of muscle beyond the electrodes no heat is produced by the warming current, though it is by stimulation. If the heating electrodes were too close to the active thermocouples these effects could produce serious errors.

(4) The muscles should be mounted under a small tension at a length about equal to that ($l_0$) in the body. They behave most regularly at this length, and preliminary stimuli should be given to make them settle down to a constant position. They must be firmly fixed at each end, so as to avoid movement during contraction. In the present experiments strong threads from the tibial tendons were tied round a fixed cross-bar just above them, so that the least possible shortening could occur.

(5) A frequency of 50 kc/s is high enough to avoid stimulation by the heating current, but not so high as to cause capacitative leaks and other troubles. It was obtained from an ordinary commercial oscillator.

*Thermopile.* A drawing of the thermopile chiefly employed in the present work was given by Abbott, Aubert & Hill (1951, Fig. 1). The central stimulating electrode was not used, it was insulated. The two dummy thermocouples at each end, and the two stimulating electrodes, help to side-track any disturbances that might be conducted from irregular temperature changes at and beyond the heating electrodes. The best published drawing of a thermopile of the present type is in Fig. 2 of a paper by Hill (1938). One can see there how heating electrodes can be fixed a few millimetres beyond the stimulating electrodes (*n*) at each end: the clamp and the muscles then have to be displaced a few millimetres to the left.

*Galvanometer.* The galvanometer used had a period of 22 msec and its deflexions were amplified photo-electrically and displayed on a cathode-ray tube (Hill, 1948). A fairly large amount of heat was produced by the muscles, from 7 to $40 \times 10^{-3}$ cal/g according to the temperature and the duration of stimulus; so considerable negative feed-back could be applied, increasing the speed of the galvanometer and stabilizing it. The galvanometer, indeed, was so fast that for the purposes of the work described here no allowance for lag was needed. To get greater long-term stability the 12 V lamp was used with 9 V, and the heaters of the head amplifier each with 4·5 V: the voltage was maintained constant by occasional adjustment with a rheostat.

*Recording.* The amplified deflexion of the galvanometer was displayed on the screen of a cathode-ray tube whose beam was modulated to give spots at intervals of 0·02–1·0 sec (as required). During heat production successive sweeps fell clear of each other, and so could all be recorded photographically upon a single piece of stationary sensitive paper 10·8 cm × 8·3 cm in size. This is convenient for measurement and storage, and the deflexion of any spot up to 75 mm was measured to 0·1 mm. The end of the stimulus was indicated by diminished brightness of the spot from this moment, and the base line was drawn by a single sweep immediately before the record was taken.

*Muscles.* A pair of sartorius muscles was used, from frog (*Rana temporaria*) or toad (*Bufo bufo*). Rather large muscles, up to 300 mg the pair, are better than smaller ones, because

their temperature falls more slowly, so the allowance for heat loss is relatively less; though muscles of 150 mg the pair give quite satisfactory results.

*Stimulation* was maximal, by means of short condenser discharges (about 0·2 msec) of frequency high enough to give a just complete tetanus.

*The Ringer's solution* used contained (mM) NaCl 115·5, KCl 2·5 and CaCl 1·8. It was buffered at pH 7·0 by phosphate 3·0 mM.

*Thermostat.* The thermopile, with its cover on, was sunk deep in a large double-walled vacuum flask (capacity 6 l.), filled either with water or with a mixture of water and ice, which was kept vigorously stirred by bubbling.

*Nitrogen.* No special precautions were necessary to ensure freedom from oxygen, since oxygen-free nitrogen is now supplied commercially. Usually the muscles, mounted on the thermopile, were kept for some time in oxygen-free Ringer's solution, with nitrogen bubbling through it, before the fluid was replaced by nitrogen.

*Analysis of heat records.* No analysis was usually needed, except the allowance for heat loss, at any time later than 1–2 sec after the end of relaxation. By then the thermopile has reached the temperature of the muscles. For an accurate determination of the early heat production, particularly at higher temperatures, an analysis is required to allow for delay in the transfer of heat from muscle to thermopile. When this was necessary it was carried out by the method of 'factors' described in earlier papers (Hill, 1949*a*, *b*).

*Osmotic effect on base line.* When a muscle is stimulated in the absence of oxygen the break-down products of activity accumulate in it and its vapour pressure falls: so water distils over to it from elsewhere and causes, by condensation, a continuing production of heat (Hill & Kupalov, 1930). The base line, therefore, shifts in a positive direction. If not allowed for this can cause a substantial error when the delayed anaerobic heat is measured over a long period. The effect, however, was small in the times involved in the present experiments, and would be in the positive, not the negative, direction.

## RESULTS

### *At 0° C*

In Fig. 1 are two curves of deflexion representing the heat produced, during and after contraction at 0° C, by a pair of toad sartorii. Details are given in the legend. The records were continued to show the early stages of the recovery heat, aerobic or anaerobic. Curve *A* is for the muscles in $O_2$, curve *B* for the same muscles in $N_2$ 57 min after $O_2$ had been removed. This was the first stimulus in $N_2$. The base lines are different, and are a long way below the figure. The deflexions plotted are millimetres as recorded, but corrected for heat loss. The sensitivities are stated in the legend. Only the last stage of the initial upward deflexion is shown in either case. In toad muscles at 0° C relaxation is not complete until 2 sec after the last shock, and the deflexions were within 0·4 mm of their maxima by 1 sec later.

After the maxima the corrected deflexions fall, reaching minima in about 37 sec in $O_2$, in about 57 sec in $N_2$ (reckoning from the beginning of stimulation). These times are so long that the fall could not be due to inequalities, within the muscles, in the spatial distribution of the heat produced during contraction and relaxation. Equalization of temperature

would not take more than about 2 sec, if such inequalities occurred (see below). In both cases the fall was clearly cut short by later positive heat production, in $O_2$ (*A*) by the usual oxidative recovery heat, in $N_2$ (*B*) by the smaller and slower delayed anaerobic heat. The negative heat in $O_2$ was 5 % of the initial heat, in $N_2$ 6·4 % of the initial heat. The experiment was continued with later stimuli in $N_2$; all the results in $N_2$ are given in Table 1.

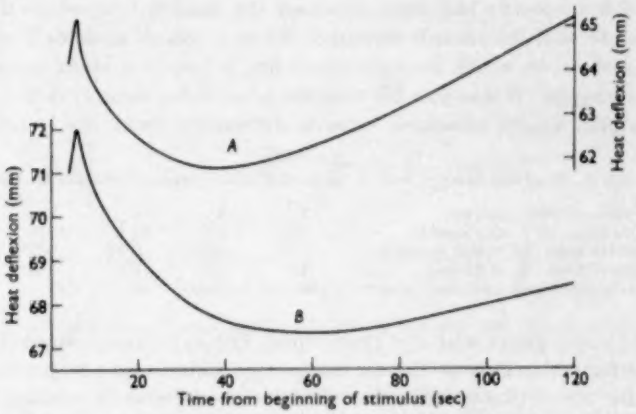



Fig. 1. Final stage of initial heat production at 0° C, followed by negative heat. Toad sartorii, in $O_2$ (*A*) and in $N_2$ 57 min (*B*). 3 sec tetanus, 10 shocks/sec.

*A*. Scale of deflexion on right, 1 mm = $0{\cdot}16 \times 10^{-3}$ cal/g. Initial heat to max. = $10{\cdot}4 \times 10^{-3}$ cal/g muscle: negative heat to min. = $0{\cdot}53 \times 10^{-3}$ cal/g muscle.

*B*. Scale of deflexion on left, 1 mm = $0{\cdot}148 \times 10^{-3}$ cal/g. Initial heat to max. = $10{\cdot}65 \times 10^{-3}$ cal/g muscle: negative heat to min. = $0{\cdot}68 \times 10^{-3}$ cal/g muscle.

TABLE 1. Negative delayed heat at 0° C after various periods without oxygen

| Time without $O_2$ (min) | 57 | 65 | 84 | 100 | 110 |
|---|---|---|---|---|---|
| Stimulus duration (sec) | 3 | 3 | 3 | 8 | 8 |
| Initial heat ($10^{-3}$ cal/g muscle) | 10·65 | 9·74 | 9·52 | 17·6 | 16·8 |
| Negative heat ($10^{-3}$ cal/g muscle) | 0·68 | 0·67 | 0·65 | 0·81 | 0·73 |
| Negative heat (% of initial) | 6·4 | 6·9 | 6·8 | 4·6 | 4·4 |

A similar experiment was made (in oxygen only) at 0° C on another pair of toad sartorii, with different durations of stimulus. All showed negative heat production after the initial maximum, but this was cut short by the oxidative recovery heat more and more as the duration of stimulus was increased. See Table 2. The time to the minimum is reckoned from the start of the stimulus. If it were reckoned from the end of the stimulus there would be a substantial decrease with longer stimuli. It has long been known (since Hartree & Hill, 1922; see also Hartree, 1932*a*) that the recovery heat production is relatively more rapid after a greater initial

heat production; so the negative heat is cut off sooner by the positive heat after a longer stimulus.

Although calculation had shown (see also below, p. 187) that any inequalities of temperature produced in a muscle during contraction and relaxation would be evened out by conduction within 1–2 sec, it was conceivable that some unknown factor had been neglected in the calculation. A few experiments, therefore, were made with single muscles, in which after a few records had been obtained the muscle was reversed on the thermopile and the records repeated. When a pair of muscles is used, the outer surface, on which the epimysium lies, is bound to be in contact with the thermopile. It was possible that the inner fibres, usually outside on the thermopile, might somehow behave differently from the outer fibres,

TABLE 2. Negative delayed heat in $O_2$ at 0° C with various durations of stimulus

| Duration of stimulus (sec) | 1 | 2 | 3 | 4 | 8 |
|---|---|---|---|---|---|
| Initial heat ($10^{-3}$ cal/g muscle) | 6·5 | 9·0 | 11·1 | 12·9 | 19·8 |
| Negative heat ($10^{-3}$ cal/g muscle) | 0·32 | 0·39 | 0·38 | 0·25 | 0·14 |
| Negative heat (% of initial) | 4·9 | 4·3 | 3·4 | 1·9 | 0·7 |
| Time to minimum (sec) | 23 | 25 | 24 | 23 | 23 |

normally in contact with the thermopile. The experiment shown in Fig. 2 was performed in $O_2$ at 0° C, on a single large sartorius of a frog with 1·2 sec tetanus. First, two records were made with the muscle arranged in the usual way: then a heating control was recorded. Next the thermopile was taken out of the thermostat and the muscle was reversed; when temperature equilibrium had been restored two more records were made, and finally another heating control.

The two records of the heat production with the epimysium inwards were averaged and corrected for heat loss. So were the two records of the heat production with the epimysium outwards. The results, in this experiment, were in fact nearly the same with the muscle one way round and the other: they were finally averaged and are shown in Fig. 2. Curve *A* gives the whole deflexion, from the start to 25 sec; curve *B* gives the later part of the deflexion, on ten times the scale, but at the same times. The negative heat occurred as usual; the minimum was at about 18 sec, after which the oxidative recovery heat began. The negative heat was $0{\cdot}37 \times 10^{-3}$ cal/g muscle, about 5·8 % of the initial heat.

In some experiments at 0° C in $O_2$ the minimum was considerably earlier, and the negative heat was correspondingly less; in these the recovery heat had evidently begun sooner, and had partly masked the negative heat. In one such experiment the negative heat in $N_2$ was several times as great as in $O_2$, because the positive delayed heat came on much more slowly in $N_2$ than in $O_2$.

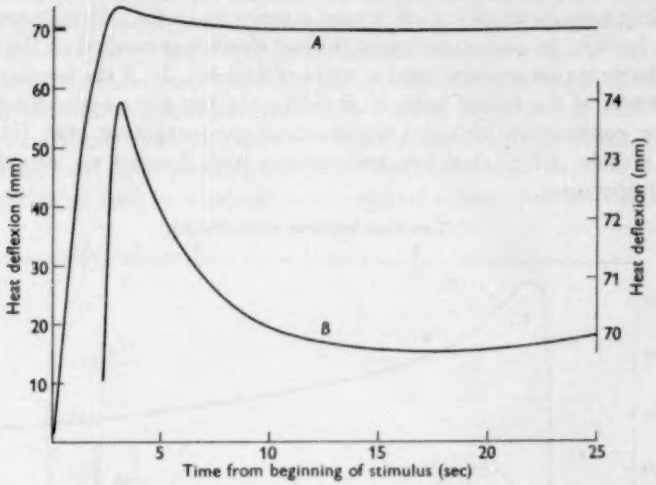



Fig. 2. Initial heat production at 0° C followed by negative heat. Single sartorius of frog in $O_2$. The deflexion, corrected for heat loss only, is the mean of 4 records, 2 with the outer surface of the muscle in contact with the thermopile, 2 with the inner surface. 1·2 sec tetanus. *A* shows the whole deflexion from the start to 25 sec, *B* shows the later portion only, on 10 times the scale; times are the same for both. Scale of deflexion, *A* on the left, *B* on the right: 1 mm = $0{\cdot}086 \times 10^{-3}$ cal/g muscle. Initial heat to max. = $6{\cdot}4 \times 10^{-3}$ cal/g muscle: negative heat to min. = $0{\cdot}37 \times 10^{-3}$ cal/g muscle.

*At room temperature*

In eight experiments with muscles in $O_2$ at temperatures from 16 to 20° C, with short stimuli, the negative heat was clearly present; but (reckoned as a fraction of the initial heat) it was smaller than at 0° C and was evidently being cut short by the earlier development of the positive recovery heat. In other experiments there was little sign of negative heat and with longer stimuli (1·2 sec and above) it never appeared. But the initial heat, with a 1·5 sec stimulus at 18° C, is rather large, usually about $50 \times 10^{-3}$ cal/g in frog muscles, about $30 \times 10^{-3}$ cal/g in toad muscles: so the positive recovery heat, the early rate of which increases more than in proportion to the initial heat, becomes too strong to allow the negative heat to appear. In twelve contractions with stimuli from 0·2 to 1·0 sec the negative heat varied from 0·1 to $1{\cdot}4 \times 10^{-3}$ cal/g muscle, from 0·9 to 3·8 % of the initial heat: it was complete (or cut short by the recovery heat) in times, reckoned from the last shock, of 1·8 sec for the longer stimuli to 9·0 sec for the shorter ones.

Figure 3 shows the negative delayed heat in two of these experiments, *A* in frog muscles at 16·7° C, *B* in toad muscles at 16·8° C. Details are given in the legend. In each experiment the last shock was marked on the record and the deflexion was analysed in units of 0·04 sec. In *A* the negative heat was 3·8 % of the initial heat, in *B* 3·5 %. In the same experiment as *A* a later contraction with a 1 sec stimulus gave negative heat $1{\cdot}4 \times 10^{-3}$ cal/g muscle (3·7 %), but two contractions with 2 and 4 sec stimuli gave practically none.

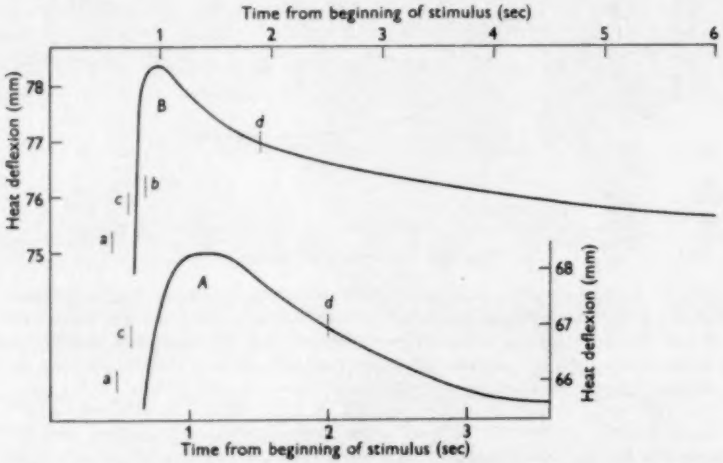



Fig. 3. Final stage of initial heat production in $O_2$ at room temperature, followed by negative heat.

*A*: frog sartorii, 16·7° C, isometric tetanus, 25 shocks, last shock (*a*) at 0·48 sec. Peak of relaxation heat (*c*) at 0·58 sec. Negative heat half complete (*d*) at 2·0 sec. Time marking below: scale of analysed deflexion on right, 1 mm = $0{\cdot}382 \times 10^{-3}$ cal/g muscle. Initial heat to max. = $26{\cdot}1 \times 10^{-3}$ cal/g muscle: negative heat to min. = $1{\cdot}03 \times 10^{-3}$ cal/g muscle.

*B*: toad sartorii, 16·8° C, isometric tetanus, 15 shocks, last shock (*a*) at 0·56 sec. Tension had fallen to zero (*b*) by 0·86 sec. Peak of relaxation heat (*c*) at 0·71 sec. Negative heat half complete (*d*) at 1·89 sec. Time marking above: scale of analysed deflexion on left, 1 mm = $0{\cdot}137 \times 10^{-3}$ cal/g muscle. Initial heat to max. = $10{\cdot}7 \times 10^{-3}$ cal/g muscle: negative heat to min. = $0{\cdot}37 \times 10^{-3}$ cal/g muscle.

In the past, when negative deflexions were observed after the initial maximum one was inclined to attribute them to inequalities, within the muscles, of the spatial distribution of the heat produced during contraction and relaxation. That such irregularities can occur was clearly shown by Hill & Howarth (1957), particularly during relaxation under large isotonic loads: and they are very evident in records of the heat production in isometric contractions with submaximal stimuli. Relaxation can be very

sudden, and if in one part of a muscle it began slightly earlier than in another the latter would be left bearing the tension, and a disproportionate amount of the elastic energy, built up during contraction, might appear as heat in that part. The fibres near the outer (skin) surface of the frog sartorius are smaller than those elsewhere (Hill, 1949*b*, Pl. 8, fig. 1, and p. 234) and if they relaxed later than the rest they would get an undue proportion of the relaxation heat. The possible effect on heat records of such an uneven distribution of heat in a muscle at the end of contraction and relaxation

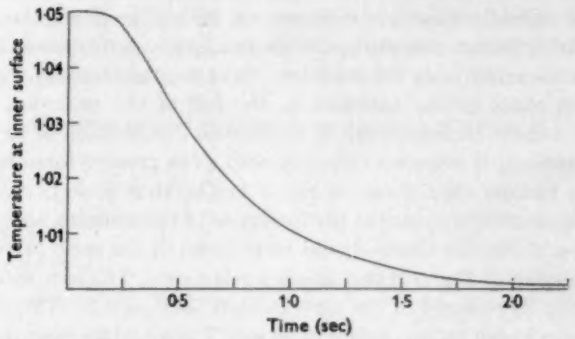



Fig. 4. To show the calculated fall of temperature of the inner surface of a muscle 0·742 mm thick after instantaneous heating of the inner 0·707 mm only, the outer 0·035 mm not being heated, except by later conduction of heat from the inner region. Initial temperature of the inner surface 1·05 (arbitrary scale): final temperature throughout 1·00.

was discussed quantitatively by Hill (1938, p. 149 and Fig. 4). The maximum delayed effect on a record would occur if a thin layer in the region of the muscle furthest from the thermopile produced no heat at all, and Fig. 4 in that paper gave a curve calculated for the hypothetical case of a muscle in which only the inner 10/11 of its cross-section produced heat. In a muscle 1·1 mm thick there would then be a 9·1 % fall of temperature at the surface of the thermopile, completed in a few seconds.

At 0° C negative heat production certainly occurs after contraction: but at higher temperatures it was necessary to look critically at the possibility that its appearance could be due to an error of the kind just described. For this purpose the curve of Fig. 4 was calculated for the case of a pair of muscles each 0·742 mm thick, which was about the thickness of the muscles used for the experiment of Fig. 3*A*. The constant $k$ for heat conduction in muscle was taken as $1{\cdot}25 \times 10^{-3}$ (Hill, 1949*b*, p. 231). Figure 4 represents an extreme example of uneven distribution, namely that of muscles in which an outermost layer, only 5 % the thickness of the rest, failed to

produce any heat. If the middle 5 % of the same muscles produced no heat, the subsequent fall at the surface would be about four times as quick.

The application of Fig. 4 to the curve of heat production in Fig. 3*A* is made as follows. The heat most likely to be unevenly distributed is that which occurs very suddenly in relaxation; and anyhow this is the latest contribution to the heat, so its uneven distribution would have the greatest effect later. The maximum rate of relaxation heat occurred at about 0·58 sec. If none of this heat appeared in the outermost 5 % of the muscle, the temperature of the inner surface would fall (according to Fig. 4) half way to its final value in a further 0·64 sec, i.e. in 1·22 sec from the start. In Fig. 3*A* the deflexion was still near its maximum at this time; it fell half way to its minimum only 0·8 sec later. In spite, therefore, of the extreme assumption made in the calculation, the fall of the deflexion after the maximum cannot be attributed to an uneven distribution of the heat.

The quantitative argument applies with even greater force in the case of muscles thinner than those of Fig. 3*A*. The time scale of the curve of Fig. 4 depends on the square of the thickness of the muscles, so for muscles of thickness $a$ mm the times should be reduced in the ratio $(a/0{\cdot}742)^2$. In the experiment of Fig. 3*B* the muscles were each 0·55 mm thick, so the times should be reduced in the ratio $(0{\cdot}55/0{\cdot}742)^2 = 0{\cdot}55$. The curve then of Fig. 4 would fall to one half in 0·35 sec. The negative heat in this contraction had scarcely begun at 0·35 sec after the peak of the relaxation heat; it reached half its full value only in about 1·18 sec from then, instead of 0·35 sec. Again, therefore, the time relations of the negative heat are much too long to be explained by an uneven spatial distribution of the relaxation heat.

It seems safe, therefore, to conclude that the early negative heat observed at higher temperatures is a genuine phenomenon, as it obviously is at 0° C. It is more difficult to observe with certainty at higher temperatures and it is cut short more quickly by ensuing positive heat production, particularly in the presence of oxygen. But it clearly is part of a general phenomenon, not characteristic only of 0° C and lack of $O_2$.

*The effect of iodoacetate on the negative delayed heat*

In order to confirm, by present methods, Hartree's results (1932*a*) on the continuing presence of the negative delayed heat after poisoning with iodoacetate (IAA), the experiments of Table 3 were made at 0° C in $N_2$ on toad sartorii. In each experiment the muscles, mounted on the thermopile, were given a preliminary soaking in ordinary oxygenated Ringer's solution at room temperature. Then at time zero the solution was changed for one containing iodoacetate (neutralized to pH 7), through which oxygen bubbled. At about 60 min the thermopile chamber was placed in the

thermostat at 0° C, at 110 min nitrogen was bubbled through the solution, at 140 min the solution was replaced by nitrogen and at 180 min the first stimulus was applied and a record made of the heat production. After that successive records were taken at intervals of 10–15 min. Finally, the temperature was raised to that of the room and a succession of shocks was applied in order to verify that the characteristic contracture of IAA poisoning set in.

The only analysis necessary for the records was to make the allowance for heat loss. When this had been done the corrected deflexion was found to remain steady for a second or two after the muscle had relaxed; then it began to decrease, reaching a minimum in about a minute, after which it started to increase slowly.

TABLE 3. The effect of iodoacetate on the negative heat of muscles at 0° C in $N_2$

| Expt. no. | 1 | | | 2 | | | 3 | | | 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stimulus (sec) | 3 | | | 5 | | | 3 | | | 3 | | |
| IAA concentration | 1/25,000 | | | 1/25,000 | | | 1/15,000 | | | 1/15,000 | | |
| Number in series | 1 | 4 | 6 | 1 | 4 | 5 | 2 | 5 | 7 | 1 | 2 | 9 |
| Initial heat ($10^{-3}$ cal/g muscle) | 8·6 | 8·3 | 7·7 | 13·7 | 12·3 | 12·1 | 8·8 | 7·9 | 6·0 | 6·3 | 7·2 | 5·9 |
| Negative delayed heat (%) | 7·9 | 11·4 | 9·2 | 7·2 | 3·6 | 4·0 | 4·4 | 4·6 | 3·9 | 4·0 | 4·0 | 4·7 |
| Complete in (sec) | 60 | 70 | 50 | 70 | 60 | 60 | 65 | 45 | 50 | 55 | 60 | 60 |

Table 3 shows that the negative delayed heat appeared in every contraction; in experiment 1 it was rather large. This confirms Hartree's conclusions, though the quantity of negative delayed heat observed in the present experiments is somewhat greater than he found.

In every one of the twelve contractions reported in Table 3 the negative deflexion was followed by a slow positive one, which was already obvious at 80–90 sec. If this later positive deflexion in the poisoned muscles were really due to the same cause as the usual positive anaerobic delayed heat, it would be impossible to explain the latter in the accepted way as the net thermal effect of the resynthesis of creatine phosphate with free energy supplied by the production of lactic acid. One was reminded, however, of the statement by Cattell, Feng, Hartree, Hill & Parkinson (1931, p. 298), on the anaerobic delayed heat of muscles poisoned with iodoacetate and stimulated at room temperature, that 'spontaneous heat production occurred in nitrogen too early to allow a reliable end to the heat record, even after the first stimulus'. Hartree also had found (1932*a*, p. 285), at 0° C, that 'for later stimuli there was a conspicuous progressive change due to subsequent positive heat occurring earlier and at a higher rate'. Moreover, Lundsgaard (1934) found that in muscles poisoned with iodoacetate at 0–2° C creatine phosphate continued to be split for some time after a 25 sec tetanus. A few experiments were made at room temperature

in the same way as those reported above for 0° C. At 13·3° C with 1/15,000 iodoacetate no negative delayed heat was found: the heat deflexion due to a stimulus was unsteady and there was a large positive shift of base line which must have been due to a permanent increase in the rate of heat production. In two experiments at 12·6 and 13·1° C with 1/25,000 iodoacetate there was clear evidence of negative delayed heat production, but it was obviously cut short by a large ensuing positive one: and after later contractions the delayed heat production was positive and persisting. In one experiment at 14° C with 1/30,000 iodoacetate there was a large positive shift of the base line, even after the first stimulus.

In view of these results at room temperature it seems rather likely that a similar permanent change in the resting heat rate took place at 0° C, too small easily to detect and occurring stepwise after each contraction. This does not at all invalidate the conclusion that a negative delayed heat production occurs after contraction in muscles poisoned with iodoacetate; indeed the reverse, for a small permanent increment in heat rate following each stimulus could only tend to mask the negative delayed heat, which may really have been greater than calculated. But the effect deprives us of any direct answer to the question of whether positive delayed anaerobic heat of the usual kind can occur in a poisoned muscle.

## DISCUSSION

The negative delayed heat is rather small, though—as seen above—easily measurable. At 0° C, where it is not so quickly cut off by the positive delayed heat, it may reach a value of nearly $10^{-3}$ cal/g, though usually it is less. This, however, may not be a true measure of the extent of the chemical reactions involved. It is possible that the observed heat absorption is the result of a coupled reaction in which the process

$$A \rightarrow B$$

occurring during contraction, with heat production $h_1$, is reversed by the free energy supplied by another reaction occurring later,

$$C \rightarrow D$$

the heat production of which is $h_2$. The net heat absorption in the two reactions together would be $(h_1 - h_2)$. If this were small compared with $h_1$ or $h_2$ the extent of the chemical reactions involved might be considerably greater than one might infer from the magnitude of the difference $(h_1 - h_2)$. In looking, therefore, for a chemical explanation of the negative delayed heat it would be reasonable to consider processes which separately could provide heat up to, say, $5 \times 10^{-3}$ cal/g.

If this heat came from the splitting of phosphate from some known, or unknown, organic precursor with a heat production of 5000 cal/mole, the

amount of phosphate produced would be 1 $\mu$mole/g muscle, and this should be detectable by present chemical methods. That at least gives the order of size of the changes to be sought, namely fractions or small multiples of 1 $\mu$mole/g muscle.

All recent chemical evidence is against the possibility that at the end of a short contraction in a normal muscle any net break-down of adenosin triphosphate (ATP) has occurred (Fleckenstein, Janke, Davies & Krebs, 1954; Mommaerts, 1954, 1955; Fleckenstein & Janke, 1957); while the experiments of Carlson & Siger (1960), with frog sartorii poisoned with 0·5 mM (1/11,000) IAA at 1–2° C, gave convincing evidence that no reduction of ATP had occurred when the muscles were frozen about 30 sec after the end of a short series of twitches and then analysed. Moreover, the experiments of Fleckenstein, Gerlach, Janke & Marmier (1960) suggested that 'the increased $O^{18}$ incorporation, at least into ATP and creatine phosphate, was more correlated with metabolic reactions in recovery... than with the contraction process itself'. The possibility also that creatine phosphate continues to be split after a contraction is eliminated by such experiments as those of Nachmansohn (1928), of Meyerhof & Nachmansohn (1930) and of Lehnartz (1931), who showed, on the contrary, that a considerable resynthesis occurs in 20 or 30 sec at room temperature. The delayed creatine phosphate break-down found by Lundsgaard (1934) was in muscles poisoned by iodoacetate: no evidence exists that it occurs in normal muscles. Finally, present thermochemical data suggest that in living muscle the heat of the reaction CrP + ADP → Cr + ATP is positive and fairly large, not negative. According to Carlson & Siger (1960) the 'physiological heat of hydrolysis' of CrP in frog muscle at 1–2° C lies between 9·6 and 11·5 kcal/mole: while the calculations of Bernhard (1956), based partly on the heat measurements of Podolsky & Morales (1956), gave 3·6 kcal/mole as the total heat produced in mammalian muscle by the splitting of ATP to adenosine diphosphate (ADP) at 37° C. Admittedly the conditions were very different in these two cases, but it is hard to believe that the physiological heat of splitting ATP in frog muscle at 0° C could be three times as great as in mammalian muscle at 37° C.

Altogether, therefore, it is impossible to attribute the negative delayed heat to a delayed synthesis of ATP by means of the free energy supplied by creatine phosphate hydrolysis. The appearance equally of the negative delayed heat in muscles poisoned with iodoacetate shows that it cannot be due to the resynthesis of creatine phosphate either by means of the free energy supplied by lactic acid formation, or in the reaction suggested by Meyerhof & Schulz (1935),

phosphopyruvic acid + creatine → creatine phosphate + pyruvic acid,

of which the heat of reaction is negative (about $-3000$ cal/mole). The possibility exists that phosphopyruvic acid might appear in poisoned muscles by some process outside the normal chain of anaerobic breakdown of carbohydrate, but even so there is no chemical evidence that creatine phosphate is restored in such muscles in the absence of $O_2$—rather the contrary, as Lundsgaard (1934) found.

It seems, therefore, that none of the processes at present known in the chemistry of muscle can be invoked to explain the delayed negative heat. Fleckenstein *et al.* (1954) produced evidence that 'inorganic phosphate was liberated during contraction from an unidentified precursor', and this might conceivably provide the key to the riddle. That unknown substances *are* liberated in muscles stimulated to fatigue was pointed out long ago by Hill & Kupalov (1930): they found that only 80 % of the fall of vapour pressure caused by stimulation to fatigue could be accounted for by known chemical bodies produced. This conclusion was confirmed by Meyerhof (1930), and by Meyerhof & Grollman (1931), measuring the depression of freezing point; also by Hill & Parkinson (1931) with muscles poisoned with iodoacetate; and it was emphasized again by Hill (1950). Nothing has been discovered in the last 30 years to fill this admitted gap in our knowledge of the chemistry of muscle. Perhaps the difficulty of explaining the negative delayed heat may provoke further experiments to fill it.

But another possibility exists, of quite a different kind, which ought to be discussed. When a muscle is stimulated at any but a very high external pH, the splitting of creatine phosphate produces a rise of pH in its interior. In itself a rise of pH, leading to an increased ionization of protein and other weak acids, would cause an absorption of heat. If the rise of pH could occur later than the splitting of creatine phosphate that caused it, the positive heat of the latter would be followed by the negative heat of the former: otherwise the second would mask the first and no negative heat would appear. But the alkalization of the interior of a muscle might be expected to occur immediately after the splitting of creatine phosphate, not during the next 60 sec (cf. Fig. 1*B* for the negative delayed heat at 0° C). Nevertheless, though unlikely, it is conceivable that the creatine phosphate might be split in some highly localized region, around which a diffusion barrier of some kind might only gradually allow its products to affect the general pH. Against this must be counted the fact that the delayed negative heat appears much more quickly at a higher temperature, which is inconsistent with simple diffusion. It is a fact, however, as shown in recent experiments by Distèche (1960), using a developed form of Dubuisson's glass-electrode system, that the pH of a muscle continues to rise for some time after a tetanus or a rapid series of twitches. At a temperature near 0° C the pH of a frog sartorius in contact with the glass

electrode went on rising after contraction for times recorded up to half a minute and had not ceased then (see his figures 63, 65, 68, 69). Earlier figures in Distèche's paper showed a similar effect in tortoise muscles. It seems extremely unlikely that this continuing rise of pH could be due to a continued splitting of creatine phosphate, for there is no evidence that this ever happens in a normal muscle: and if it did it would not solve the present problem, for then there would be a production, not an absorption, of heat.

Either therefore the splitting of creatine phosphate during contraction affects the general pH of a muscle only gradually, a rather unlikely hypothesis, or some unknown chemical reaction is involved. Without more chemical evidence it is impossible to decide between these alternatives, or about the origin of the delayed negative heat.

SUMMARY

1. The early delayed absorption of heat following a short contraction, described by Hartree in 1932, has been re-examined.

2. It occurs in the presence as well as in the absence of oxygen, and at room temperature as well as at 0° C.

3. It is cut short by the ordinary positive delayed heat, aerobic or anaerobic, that follows it.

4. It may be as large as 1 mcal/g muscle after a short contraction, or 5–10 % of the initial heat.

5. In the absence of oxygen, at 0° C after a short tetanus it may continue for 50–60 sec: after a long tetanus it is masked by the anaerobic delayed heat coming on earlier. At a higher temperature it is over sooner, as it is in the presence of oxygen.

6. It occurs equally in muscles poisoned with iodoacetate.

7. It is not possible to explain it in terms of chemical reactions known as yet to take place after contraction.

8. It may be associated with the rise of pH recently observed by Distèche to occur gradually after a series of twitches or a tetanus.

My thanks are due to Mr R. C. Woledge for his skilled assistance, during his tenure of a M.R.C. scholarship.

## APPENDIX

### BY R. C. WOLEDGE

The experiments reported in the preceding paper by Hill were made during the months of August to December. Additional evidence for the negative delayed heat has now been found in a series of experiments made, for another purpose, during May with the sartorii of spring frogs. In these

experiments records were made of the heat production in oxygen during and after tetani of various durations, at 0° C and at 17° C. At 0° C the negative delayed heat was similar to that found with autumn frogs. But, unexpectedly, the absorption of heat after a tetanus at 17° C was sometimes larger and more prolonged than with tetani of the same duration in the earlier series of experiments. There appears to be a seasonal difference in the metabolic processes of spring frogs which either increases the intensity of the negative delayed heat or slows the onset of the positive recovery heat which tends to mask it.

The results of one experiment, in which the negative heat was particularly large, are given in Table 1, which shows the amount and time course of the negative heat observed after tetani of various durations. 'Half time' means the time from the end of the tetanus to the half completion of the observed negative deflexion; 'minimum' is the time to completion of the deflexion.

TABLE 1. Negative delayed heat in $O_2$ at 17° C with various durations of tetanus

| | | | | | |
|---|---|---|---|---|---|
| Tetanus duration (sec) | 0·2 | 0·4 | 0·8 | 1·6 | 3·2 |
| Initial heat (mcal/g) | 13·9 | 19·2 | 32·4 | 53·5 | 97·0 |
| Negative heat (mcal/g) | 0·84 | 1·5 | 2·0 | 1·9 | 0·6 |
| Negative heat as % of initial | 6·1 | 7·8 | 6·0 | 3·6 | 0·6 |
| Half time (sec) | 2·0 | 1·7 | 1·7 | 1·3 | 1·0 |
| Minimum (sec) | 9 | 8 | 8 | 4 | 2 |

The thickness of each muscle in this experiment was 0·67 mm. The curve of Fig. 4 in the preceding paper can thus be applied in this case by multiplying the times by 0·75. Its application shows that any unevenly produced heat would have distributed itself within about 1 sec. The maximum rate of relaxation heat at 17° C comes about 0·1 sec after the last shock, so that any effect of its uneven distribution would be over by 1·1 sec after the last shock. In the shorter tetani the negative heat was only beginning at this time.

In another experiment (at 16·5° C) the quantities of heat absorbed after the end of the tetani were about 70% of those in the experiment just referred to, but the records were not continued long enough to observe the end of the heat absorption. Again there was a definite negative heat after a 1·6 sec tetanus and some sign of heat absorption even after a 3·2 sec tetanus. In a third experiment the greatest negative heat was 2·1% (after a 0·8 sec tetanus), there was a heat absorption of 0·9% after a 1·6 sec tetanus but none after a 3·2 sec tetanus. In one further experiment there was no sign at all of the negtive heat.

The negative delayed heat at room temperature is evidently a rather variable phenomenon, as was stated in the preceding paper. But it is clear that, at room temperature, the heat absorption is sometimes particularly

evident in the muscles of spring frogs. These observations make it even more certain that the negative delayed heat really is a general phenomenon occurring both at 0° C and at room temperature, in oxygen as well as in nitrogen. Doubtless even better evidence of the effect at room temperature, were that required, could be obtained by making the experiments in nitrogen, when it would not be masked by the onset of the oxidative recovery heat.

## REFERENCES


ABBOTT, B. C., AUBERT, X. M. & HILL, A. V. (1951). The absorption of work by a muscle stretched during a single twitch or a short tetanus. *Proc. Roy. Soc.* B, **139**, 86–104.

BERNHARD, S. A. (1956). Ionization constants and heats of tris(hydroxymethyl)aminomethane and phosphate buffers. *J. biol. Chem.* **218**, 961–969.

BUGNARD, L. (1934). The relation between total and initial heat in single muscle twitches. *J. Physiol.* **82**, 509–519.

CARLSON, F. D. & SIGER, A. (1960). The mechanochemistry of muscular contraction. *J. gen. Physiol.* **44**, 33–60.

CATTELL, McK., FENG, T. P., HARTREE, W., HILL, A. V. & PARKINSON, J. L. (1931). Recovery heat in muscular contraction without lactic acid formation. *Proc. Roy. Soc.* B, **108**, 279–301.

DISTÈCHE, A. (1960). Contribution à l'étude des échanges d'ions hydrogène au cours du cycle de la contraction musculaire. *Mém. Acad. R. Belg.* **32**, 1–169.

FLECKENSTEIN, A., GERLACH, E., JANKE, J. & MARMIER, Y. Z. (1960). Die Inkorporation von markiertem Sauerstoff aus Wasser in die ATP-, Kreatinphosphat- und Orthophosphat-Fraktion intakter Muskeln bei Ruhe, tetanischer Reizung und Erholung. *Pflüg. Arch. ges. Physiol.* **271**, 75–104.

FLECKENSTEIN, A. & JANKE, J. (1957). Der Austausch von radioaktivem $^{32}$P-markiertem Orthophosphat mit dem $P^{\alpha}$, $P^{\beta}$ und $P^{\gamma}$ von ATP und mit Kreatinphosphat bei Muskelruhe, Temperaturvariation und elektrischer Reizung. *Pflüg. Arch. ges. Physiol.* **265**, 237–263.

FLECKENSTEIN, A., JANKE, J., DAVIES, R. E. & KREBS, H. A. (1954). Chemistry of muscle contraction. *Nature, Lond.*, **174**, 1081.

FURUSAWA, K. & HARTREE, W. (1926). The anaerobic delayed heat production in muscle. *J. Physiol.* **62**, 203–210.

HARTREE, W. (1931). The analysis of the initial heat production of muscle. *J. Physiol.* **72**, 1–26.

HARTREE, W. (1932*a*). The analysis of the delayed heat production of muscle. *J. Physiol.* **75**, 273–287.

HARTREE, W. (1932*b*). A negative phase in the heat production of muscle. *J. Physiol.* **77**, 104–110.

HARTREE, W. & HILL, A. V. (1922). The recovery heat production in muscle. *J. Physiol.* **56**, 367–381.

HARTREE, W. & HILL, A. V. (1928). The anaerobic delayed heat production after a tetanus. *Proc. Roy. Soc.* B, **103**, 207–217.

HILL, A. V. (1938). The heat of shortening and the dynamic constants of muscle. *Proc. Roy. Soc.* B, **126**, 136–195.

HILL, A. V. (1939). Recovery heat in muscle. *Proc. Roy. Soc.* B, **127**, 297–307.

HILL, A. V. (1948). Moving-coil galvanometers of short period and their amplification. *J. sci. Instrum.* **25**, 225–229.

HILL, A. V. (1949*a*). The numerical analysis of records to eliminate time lag. *J. sci. Instrum.* **26**, 56–57.

HILL, A. V. (1949*b*). Myothermic methods. *Proc. Roy. Soc.* B, **136**, 228–241.

HILL, A. V. (1950). A challenge to biochemists. *Biochim. biophys. acta*, **4**, 4–11.

HILL, A. V. & HOWARTH, J. V. (1957). Alternating relaxation heat in muscle twitches. *J. Physiol.* **139**, 466–473.

HILL, A. V. & KUPALOV, P. S. (1930). The vapour pressure of muscle. *Proc. Roy. Soc.* B, **106**, 445–477.

HILL, A. V. & PARKINSON, J. L. (1931). Heat and osmotic change in muscular contraction without lactic acid formation. *Proc. Roy. Soc.* B, **108**, 148–156.

HILL, D. K. (1940). The anaerobic recovery heat production of frog's muscle at 0° C. *J. Physiol.* **98**, 460–466.

LEHNARTZ, E. (1931). Ueber die Nachträgliche Milchsäurebildung beim Tetanus und bei der Zuckung isolierten Froschmuskeln. *Hoppe-Seyl. Z.* **197**, 55–89.

LUNDSGAARD, E. (1931). Ueber die Energetik der anaeroben Muskelkontraktion. *Biochem. Z.* **233**, 322–343.

LUNDSGAARD, E. (1934). Phosphagen- und Pyrophosphatumsatz in jodessigsäurevergifteten Muskeln. *Biochem. Z.* **269**, 308–328.

MEYERHOF, O. (1930). Ueber die Aenderung des osmotischen Drucks des Muskels bei Ermüdung und Starre. *Biochem. Z.* **226**, 1–15.

MEYERHOF, O. & GROLLMAN, A. (1931). Weitere Versuche über den Zusammenhang zwischen chemischem Umsatz und osmotischer Drucknahme im Muskel. *Biochem. Z.* **241**, 23–35.

MEYERHOF, O. & NACHMANSOHN, D. (1930). Ueber die Synthese der Kreatinphosphorsäure im lebenden Muskel. *Biochem. Z.* **222**, 1–20.

MEYERHOF, O. & SCHULZ, W. (1935). Ueber die Energieverhältnisse bei der enzymatischen Milchsäurebildung und der Synthese der Phosphagene. *Biochem. Z.* **281**, 292–305.

MOMMAERTS, W. F. H. M. (1954). Is adenosine triphosphate broken down during a single muscle twitch? *Nature, Lond.*, **174**, 1083–1084.

MOMMAERTS, W. F. H. M. (1955). *Amer. J. Physiol.* **182**, 587–593.

NACHMANSOHN, D. (1928). Ueber den Zerfall der Kreatinphosphorsäure in Zusammenhang mit der Tätigkeit des Muskels. *Biochem. Z.* **196**, 73–97.

PODOLSKY, R. J. & MORALES, M. F. (1956). The enthalpy change of adenosine triphosphate hydrolysis. *J. biol. Chem.* **218**, 945–959.

*J. Physiol.* (1961), **158**, *pp.* 197–205
*With 6 text-figures*
*Printed in Great Britain*



# RELATIONSHIP BETWEEN PULMONARY ARTERIAL PRESSURE AND IMPULSE ACTIVITY IN PULMONARY ARTERIAL BARORECEPTOR FIBRES

BY J. C. G. COLERIDGE AND C. KIDD
*From the Department of Physiology, School of Medicine, University of Leeds*

(*Received* 28 *April* 1961)

There is now good physiological evidence for the presence of baroreceptor endings in the wall of the extrapulmonary parts of the pulmonary artery of the cat (Swan & Whitteridge, 1956; Bianconi & Green, 1959) and the dog (Coleridge & Kidd, 1960*a*; Coleridge, Kidd & Sharp, 1961).

The present study is an attempt to define the relationship between the impulse activity recorded from the afferent vagal fibres of these receptors and the pressure in the pulmonary artery. A preliminary report has already been published (Coleridge & Kidd, 1960*b*).

## METHODS

Experiments were performed on dogs anaesthetized with chloralose (0·08 g/kg intravenously) or, after premedication with morphine sulphate (3 mg/kg subcutaneously), with 0·25 ml./kg (intravenously) of a 1:1 mixture of Dial Compound (allobarbitone-urethane, Ciba) and sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd.) solutions.

A tracheal cannula was inserted and the lungs were ventilated by a Starling 'Ideal' pump. The sternum was split in the mid line. Ligatures were placed loosely round the inferior vena cava just above the diaphragm, the origin of the main pulmonary artery and both lung roots.

Pulmonary arterial pressure was recorded by an optical manometer (Coleridge & Linden, 1954; membrane 0·003 in. (0·076 mm) thick, diameter 9 mm) connected to a wide-bore metal cannula inserted into the central end of one of the hilar branches of the pulmonary artery. The natural frequency of the system with cannula, lead tubing and cock attached was 120–150 c/s. Respiration was recorded with an optical manometer attached to a side arm on the tracheal cannula.

Action potentials were recorded from pulmonary arterial baroreceptor fibres dissected from the right or left cervical vagus nerves. Details of the recording techniques and methods of identification of pulmonary arterial baroreceptors have been described previously (Coleridge & Kidd, 1960*a*; Coleridge *et al.* 1961).

Alterations in pulmonary arterial pressure were brought about by infusing blood, saline (NaCl, 0·9 g/100 ml.) or dextran (6 g/100 ml., in NaCl solution 0·9 g/100 ml.; 'Dextraven', Benger Laboratories Ltd.) at body temperature into the femoral vein, or by bleeding the animal from a femoral artery. The pressure was also varied by occlusion and release of the inferior vena cava.

The relationship between baroreceptor activity and pulmonary arterial pressure was also

examined after the pulmonary artery (the main pulmonary trunk and the main right and left branches) had been isolated from the rest of the circulation. The fibre from a pulmonary arterial baroreceptor was retained on the recording electrodes and the animal was rapidly bled to death. The right ventricle was opened. A wide-bore cannula was inserted through the ventricle into the main pulmonary artery and secured by a ligature; the cannula was then connected via a cock to a pressure bottle filled with 0·9 % NaCl. All lobar arteries were ligated, except the vessel from which the pressure in the pulmonary artery was recorded. The pressure in the isolated artery could then be raised by opening the cock. A pulsatile pressure was obtained by squeezing the rubber tubing leading from the pressure bottle to the cannula. The experimental observations were completed within a few minutes of arrest of the circulation, but alterations in impulse activity could be produced by varying the pressure in the isolated artery for more than 30 min after death of the animal.

## RESULTS

Observations were made on eleven afferent vagal fibres in which the impulse activity was reduced or abolished when pulmonary arterial pressure was lowered by occlusion of the origin of the main pulmonary artery, and increased when the pressure was raised by occlusion of the lung roots. In each experiment mechanical stimulation of the vessel wall with a fine probe, after death of the animal, confirmed that the receptor endings were situated in the extrapulmonary parts of the pulmonary artery. Impulse activity in these fibres showed the familiar pattern associated with arterial baroreceptors (Fig. 1). The discharge began 80–140 msec after the Q wave of the e.c.g. as the pressure in the pulmonary artery approached its systolic peak, and continued into early diastole.

### *Effect of alterations in pulmonary arterial pressure*

In ten of the eleven fibres examined the discharge synchronous with the systolic pressure pulse in the pulmonary artery gradually diminished and finally disappeared when pulmonary arterial pressure was lowered progressively by occluding the inferior vena cava (Fig. 2) or by bleeding the animal (Fig. 3). Impulse activity returned when the pressure was restored. In this way it was possible to determine the threshold of the receptors, i.e. the minimum pressure (systolic/diastolic) which was effective in eliciting a single impulse during ventricular systole.

The records depicted in Fig. 2 illustrate the effects produced by occlusion and release of the inferior vena cava. The vessel was occluded between *A* and *B* and the discharge was abolished. Record *B* was taken as the occluding ligature was slowly released. In the first pulmonary arterial pressure pulse (20/13 mm Hg) in *B* the receptor was silent during ventricular systole. In the second (24/9 mm Hg) and third (24/11 mm Hg) cycles a single spike can be seen at the peak of the pressure pulse; thereafter the discharge increased as the pressure rose with increased flow to the heart.

With reduced filling, e.g. after haemorrhage or occlusion of the inferior vena cava, baroreceptors in the right branch of the pulmonary artery sometimes showed a brief discharge in time with atrial systole (e.g. Fig. 2). In the present experiments this activity consisted of one or two impulses only, although occasionally it may be prolonged throughout atrial systole to form a conspicuous volley (Coleridge & Kidd, 1960*a*, Fig. 8). Since these adventitious discharges were not related to any corresponding event in the

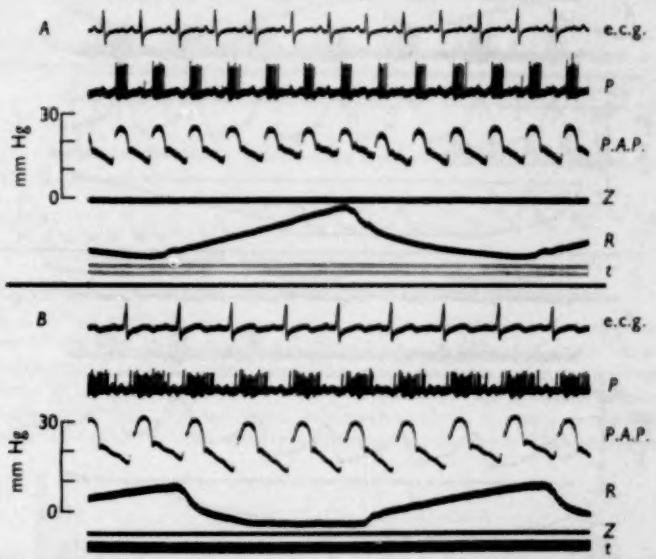



Fig. 1. Simultaneous recording of pressure in the pulmonary artery and impulse activity in pulmonary arterial baroreceptor fibres. Records *A* and *B* were made in different animals. In this and subsequent figures the following abbreviations are used: e.c.g., electrocardiogram; *P*, potentials recorded from pulmonary arterial baroreceptor fibre; *P.A.P.*, pressure in pulmonary artery (mm Hg); *Z*, zero reference line for optical manometer; *R*, tracheal pressure (upstroke representing inflation); *t*, time trace (1/50 sec).

pulmonary arterial pressure trace, and were thought to be due to distortion caused by right atrial contraction, they have been ignored for the purposes of determining threshold.

In observations on ten pulmonary arterial baroreceptors the threshold pressures (systolic/diastolic) were within the range 16–25/7–13 mm Hg. The threshold of a particular receptor sometimes varied slightly; in one instance, for example, the threshold gradually rose from 19/7 to 25/10 mm Hg over a period of 2 hr.

Since receptors in the pulmonary artery could be identified only when they were active, repeated infusions of blood or dextran solution were given to maintain a high pulmonary arterial pressure during the search for their afferent fibres in the vagus nerves. Nevertheless, the possibility cannot be excluded that receptors with a high threshold may have been overlooked because they were inactive under the conditions of our experiments.

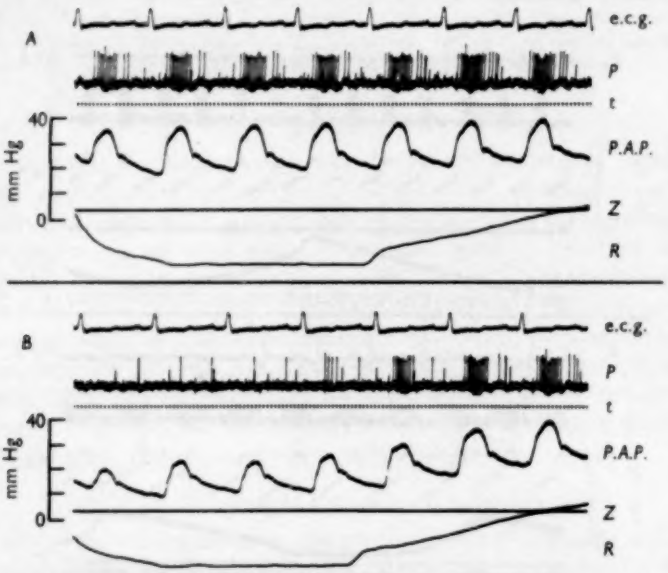



Fig. 2. Determination of threshold of pulmonary arterial baroreceptor (larger spikes). *A*, before occlusion of inferior vena cava; between *A* and *B* a ligature was tightened round the vena cava just above the diaphragm; the ligature was slowly released during *B*. In *B* note the one or two spikes in time with the P wave of the e.c.g.; the receptor was in the right pulmonary artery (see text).

It was not possible to determine the threshold of one of the eleven baroreceptors investigated. Although activity in this fibre was at first reduced when pulmonary arterial pressure was lowered, with further reduction in pressure the cardiovascular pattern of activity was gradually replaced by a continuous discharge. Previous workers have observed a continuous discharge in some baroreceptor endings in the carotid sinus at low pressures (e.g. Landgren, 1952); the activity was thought to be due to deformation of the vessel wall at these low pressures.

When pulmonary arterial pressure was raised in steps above the threshold (Fig. 3*B*) the baroreceptors signalled each rise in pressure with an

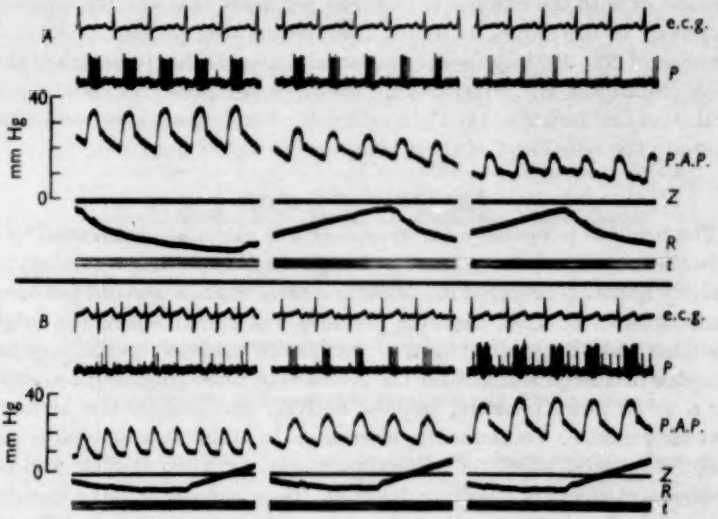



**Fig. 3.** Effect of alterations in pulmonary arterial pressure on pulmonary baroreceptor discharge. Records *A* and *B* were obtained in different animals. *A*, pulmonary arterial pressure (previously raised by infusion of dextran) was gradually lowered by haemorrhage. *B*, pulmonary arterial pressure was gradually raised by transfusion of blood previously taken from the animal. In *B*, note a second active fibre (smaller spikes) firing irregularly in the first and continuously in the third record.

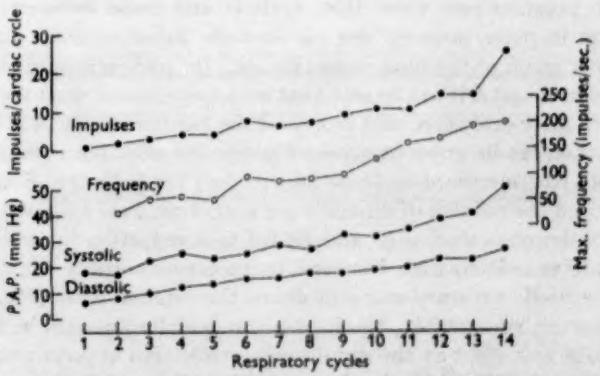



**Fig. 4.** Relationship between pulmonary baroreceptor discharge and pulmonary arterial pressure during intravenous infusion of blood (the pressure had previously been lowered by bleeding). The points plotted extend over the duration of 14 consecutive respiratory cycles. In each respiratory cycle measurements were made on the cardiac cycle at the peak of inflation. From above downwards: number of impulses per cardiac cycle; maximum frequency of discharge (impulses/sec); systolic and diastolic pulmonary arterial pressure (mm Hg).

increase in both the number of impulses per heart beat and the maximum frequency of discharge, as in the example plotted in Fig. 4. Peak frequencies of 200–300 impulses/sec occurred with pulsatile pressures of about 45–50/20–25 mm Hg. Attempts to obtain higher pressures usually led to dilatation and failure of the right ventricle. Consequently we were unable to study the behaviour of these receptors at higher pressures.

*Effect of pulsatile pressure*

The firing of pulmonary baroreceptors was obviously influenced by the pulsatile nature of the pressure changes in the pulmonary artery; for activity normally occurred in systole and early diastole and did not become continuous even when diastolic pressure was raised above the original systolic level. In general, for a given systolic pressure, pulmonary baroreceptor discharge was greater the greater the pulse pressure; conversely, for a given pulse pressure, impulse activity was greater the higher the systolic pressure. Consequently alterations in pulmonary arterial pressure had the greatest effect on baroreceptor activity when systolic and pulse pressures changed in the same direction. Thus impulse activity invariably increased when both systolic and pulse pressures rose (Figs. 2, 3*B*); while activity was always reduced when systolic and pulse pressures fell together (Fig. 3*A*).

By contrast, alterations in receptor activity were less predictable when systolic and pulse pressures changed in opposite directions. Thus systolic pressure always rose when the lung roots were occluded, but sometimes diastolic pressure rose more than systolic and pulse pressure fell. This reduction in pulse pressure was particularly liable to occur during prolonged occlusion of the lung roots (Fig. 5). By comparison of the records depicted in *A* and *B* it can be seen that both systolic and diastolic pressures increased after occlusion, and in two of the cardiac cycles in *B* there was a large increase in pulse pressure. Clearly the effective stimulus to the receptors had increased in these two cycles; for both the discharge frequency and the number of impulses per heart beat were augmented. However, continued occlusion (*C* and *D*) led to a reduction in pulse pressure which was associated with decreased baroreceptor activity (Fig. 5*D*), even though systolic pressure was still above the original level (Fig. 5*A*). In this instance, presumably, the increase in systolic pressure in *D* as compared to *A* was offset by the simultaneous reduction in pulse pressure and, on balance, the effective stimulus was reduced.

The relationship between pulsatile pressure and receptor discharge was further investigated in experiments on the isolated pulmonary artery (see Methods). The effects of pulsatile and non-pulsatile distension of the isolated artery are compared in the records shown in Fig. 6, where in *A* a

pulsatile pressure set up a discharge, but a steady pressure, greater than the previous systolic pressure, produced no discharge at all (*B*). Return to pulsatile distension (*C*) showed that the receptor was still active. The discharge shown in Fig. 6*A* and *C* appears sparse by comparison with the

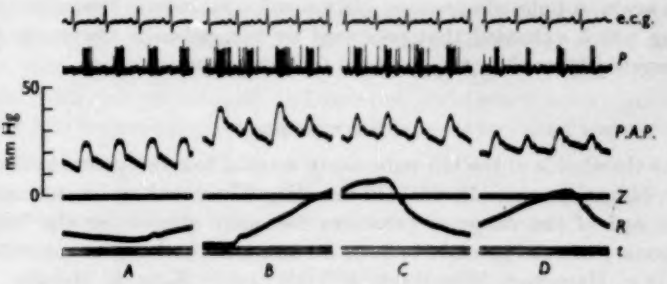



Fig. 5. Effect of occlusion of lung roots on pulmonary arterial pulse pressure and pulmonary baroreceptor activity. *A*, before occlusion; *B*, *C* and *D* during occlusion of the lung roots. Intervals of approximately 2, 3 and 1 sec between records *A* and *B*, *B* and *C*, and *C* and *D* respectively.

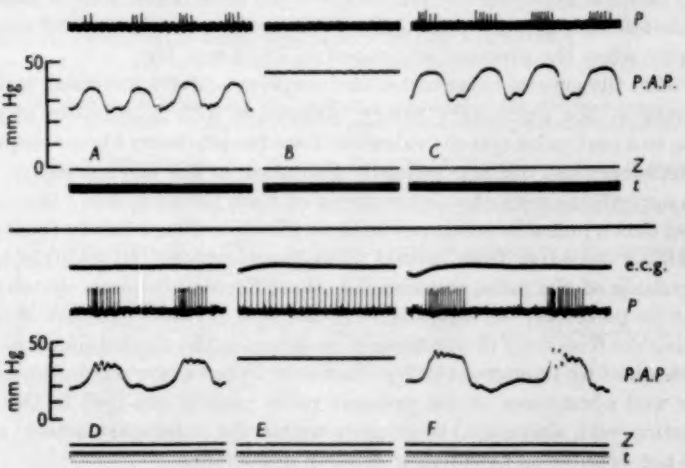



Fig. 6. Comparison of the effects of pulsatile and steady pressure in the isolated pulmonary artery on pulmonary baroreceptor discharge. Records *A–C* and *D–F* were made in different animals (note different camera speeds). In *A*, *C*, *D* and *F* the artery was distended with a pulsatile pressure, and in *B* and *E* with a steady pressure. Note the variations in impulse frequency with the oscillations at the peak of the pressure waves in *D* and *F*. The zero reference lines in *A–C* have been retouched.

baroreceptor activity recorded with normal circulation through the intact pulmonary artery (see previous figures). This was probably due to the small pulse pressure and the comparatively slow rate of change of pressure. The more rapid ascent of the pressure pulse in experiments on another fibre (Fig. 6*D–F*) was associated with a much higher impulse frequency. Once again, a pulsatile pressure (*D*) caused a maximum frequency of discharge which exceeded that produced by non-pulsatile distension (*E*) at a pressure higher than the previous systolic pressure.

## DISCUSSION

The thresholds of the ten pulmonary arterial baroreceptors investigated lay within the range 16–25/7–13 mm Hg. These values lie towards the lower end of the range of pressures variously quoted for the 'normal' pulmonary arterial pressure in both the anaesthetized and unanaesthetized dog (e.g. Hamilton, Woodbury & Vogt, 1939; Katz & Steinitz, 1940; Hürlimann & Wiggers, 1953; Simmons, Hemingway & Ricchiuti, 1958). Hence these particular receptors were active at the pressures normally prevailing in the pulmonary circulation of the dog. Moreover, there was a conspicuous increase in baroreceptor activity with a comparatively small increase in pressure above the threshold level. Thus, with a pulsatile circulation through the pulmonary artery, all receptors showed marked activity when the pressure was raised to 35/15 mm Hg.

Within the general observation that impulse activity increased with the pressure in the pulmonary artery, distension with a pulsatile pressure rising to a particular systolic value was found to produce a higher frequency of discharge than did non-pulsatile distension at the same pressure. This is in accordance with the observations of Ead, Green & Neil (1952), who found that a pulsatile pressure was more effective than a steady pressure in evoking a discharge from carotid sinus baroreceptors. In addition to the magnitude of the pulse pressure, i.e. the difference between systolic and diastolic pressures, the significance of the rate of rise of pressure in determining the frequency of discharge of receptors in the carotid sinus has been emphasized by Landgren (1952). However, in our experiments the magnitude and abruptness of the pressure pulse usually changed in the same direction with alterations in pressure within the pulmonary artery, and it was not possible to assess their relative importance.

## SUMMARY

1. The relationship between pulmonary arterial pressure and the impulse activity recorded from pulmonary arterial baroreceptor fibres has been investigated in anaesthetized dogs.

2. The thresholds of ten pulmonary arterial baroreceptors were within the range 16–25/7–13 mm Hg (systolic/diastolic). These values lie towards the lower end of the range of pressures variously quoted for the normal pulmonary arterial pressure in the dog.

3. The baroreceptors signalled each rise in pressure above the threshold with an increase in the frequency of discharge and the number of impulses per cardiac cycle. Frequencies of 200–300 impulses/sec occurred during systole with pressures of about 45–50/20–25 mm Hg.

4. In both the intact and the isolated pulmonary artery pulsatile pressure was a more effective stimulus to the receptors than was a steady pressure.

We are indebted to Dr W. J. O'Connor for valuable criticism. We wish to thank Mrs Barbara James and Mr E. S. Stainthorpe for technical assistance.

## REFERENCES

BIANCONI, R. & GREEN, J. H. (1959). Pulmonary baroreceptors in the cat. *Arch. ital. Biol.* **97**, 305–315.

COLERIDGE, J. C. G. & KIDD, C. (1960*a*). Electrophysiological evidence of baroreceptors in the pulmonary artery of the dog. *J. Physiol.* **150**, 319–331.

COLERIDGE, J. C. G. & KIDD, C. (1960*b*). The response of pulmonary arterial baroreceptors to alterations in pulmonary arterial pressure. *J. Physiol.* **152**, 33–34*P*.

COLERIDGE, J. C. G., KIDD, C. & SHARP, J. A. (1961). The distribution, connexions and histology of baroreceptors in the pulmonary artery, with some observations on the sensory innervation of the ductus arteriosus. *J. Physiol.* **156**, 591–602.

COLERIDGE, J. C. G. & LINDEN, R. J. (1954). The measurement of effective atrial pressure. *J. Physiol.* **126**, 304–318.

EAD, H. W., GREEN, J. H. & NEIL, E. (1952) A comparison of the effects of pulsatile and non-pulsatile blood flow through the carotid sinus on the reflexogenic activity of the sinus baroceptors in the cat. *J. Physiol.* **118**, 509–519.

HAMILTON, W. F., WOODBURY, R. A. & VOGT, E. (1939). Differential pressures in the lesser circulation of the unanesthetized dog. *Amer. J. Physiol.* **125**, 130–141.

HÜBLIMANN, A. & WIGGERS, C. J. (1953). The effects of progressive general anoxia on the pulmonary circulation. *Circulation Res.* **1**, 230–237.

KATZ, L. N. & STEINITZ, F. S. (1940). Pulmonary arterial pressure in experimental renal hypertension. *Amer. J. Physiol.* **128**, 433–439.

LANDGREN, S. (1952). On the excitation mechanism of the carotid baroceptors. *Acta physiol. scand.* **26**, 1–34.

SIMMONS, D. H., HEMINGWAY, A. & RICCHIUTI, N. (1958). Acute circulatory effects of pneumothorax in dogs. *J. appl. Physiol.* **12**, 255–261.

SWAN, A. A. B. & WHITTERIDGE, D. (1956). Baroreceptor fibres from the pulmonary artery. *Abstr. XX int. physiol. Congr.* 867–868.

THE JOURNAL OF PHYSIOLOGY, SEPTEMBER 1961

VOL. 158, NO. 1

## CONTENTS

PAGE

CROSFILL, M. L. AND WIDDICOMBE, J. G. PHYSICAL CHARACTERISTICS OF THE CHEST AND LUNGS AND THE WORK OF BREATHING IN DIFFERENT MAMMALIAN SPECIES . . . . . 1

ARMETT, CHRISTINE J. AND HUNSPERGER, R. W. EXCITATION OF RECEPTORS IN THE PAD OF THE CAT BY SINGLE AND DOUBLE MECHANICAL PULSES . . . . . 15

TORRANCE, H. B. THE CONTROL OF THE HEPATIC ARTERIAL CIRCULATION 39

FISHER, R. B. AND YOUNG, D. A. B. DIRECT DETERMINATION OF EXTRACELLULAR FLUID IN THE RAT HEART . . . . . 50

ZACHARIAH, P. CONTRACTILITY AND SUGAR PERMEABILITY IN THE PERFUSED RAT HEART . . . . . 59

FISHER, R. B. AND ZACHARIAH, P. THE MECHANISM OF THE UPTAKE OF SUGARS BY THE RAT HEART AND THE ACTION OF INSULIN ON THIS MECHANISM . . . . . 73

FISHER, R. B. AND WILLIAMSON, J. R. THE OXYGEN UPTAKE OF THE PERFUSED RAT HEART . . . . . 86

FISHER, R. B. AND WILLIAMSON, J. R. THE EFFECTS OF INSULIN, ADRENALINE AND NUTRIENTS ON THE OXYGEN UPTAKE OF THE PERFUSED RAT HEART . . . . . 102

CAMPBELL, ROSA M., CUTHBERTSON, D. P., MACKIE, W., MCFARLANE, A. S., PHILLIPSON, A. T. AND SUDSANEH, SAOVANEE. PASSAGE OF PLASMA ALBUMIN INTO THE INTESTINE OF THE SHEEP . . 113

MAGEE, D. F. AN INVESTIGATION INTO THE EXTERNAL SECRETION OF THE PANCREAS IN SHEEP . . . . . 132

BREBNER, D. F. AND KERSLAKE, D. MCK. THE EFFECT OF CYCLICAL HEATING OF THE FRONT OF THE TRUNK ON THE FOREARM SWEAT RATE . 144

DÉLÉZE, J. B. THE MECHANICAL PROPERTIES OF THE SEMITENDINOSUS MUSCLE AT LENGTHS GREATER THAN ITS LENGTH IN THE BODY . . 154

STOPP, PHYLLIS E. AND WHITFIELD, I. C. UNIT RESPONSES FROM BRAIN-STEM NUCLEI IN THE PIGEON . . . . . 165

HILL, A. V. THE NEGATIVE DELAYED HEAT PRODUCTION IN STIMULATED MUSCLE. WITH AN APPENDIX BY R. C. WOLEDGE . . . . . 178

COLERIDGE, J. C. G. AND KIDD, C. RELATIONSHIP BETWEEN PULMONARY ARTERIAL PRESSURE AND IMPULSE ACTIVITY IN PULMONARY ARTERIAL BARORECEPTOR FIBRES . . . . . 197

*Printed in Great Britain at the University Press, Cambridge*
*(Brooke Crutchley, University Printer)*